

1 **Ability of nucleoside-modified mRNA to encode HIV-1 envelope trimer nanoparticles**

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59 **SUMMARY**

60 The success of nucleoside-modified mRNAs in lipid nanoparticles (mRNA-LNP) as COVID-19  
61 vaccines heralded a new era of vaccine development. For HIV-1, multivalent envelope (Env)  
62 trimer protein nanoparticles are superior immunogens compared to trimers alone for priming of  
63 broadly neutralizing antibody (bnAb) B cell lineages. The successful expression of complex  
64 multivalent nanoparticle immunogens with mRNAs has not been demonstrated. Here we show  
65 that mRNAs can encode antigenic Env trimers on ferritin nanoparticles that initiate bnAb precursor  
66 B cell expansion and induce serum autologous tier 2 neutralizing activity in bnAb precursor  $V_H +$   
67  $V_L$  knock-in mice. Next generation sequencing demonstrated acquisition of critical mutations, and  
68 monoclonal antibodies that neutralized heterologous HIV-1 isolates were isolated. Thus, mRNA-  
69 LNP can encode complex immunogens and are of use in design of germline-targeting and  
70 sequential boosting immunogens for HIV-1 vaccine development.

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72 **KEYWORDS:**

73 mRNA, lipid nanoparticles, mRNA-LNP, HIV-1, vaccine, broadly neutralizing antibodies, knock-in  
74 mice

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## 85 INTRODUCTION

86 The recent success of nucleoside-modified mRNA COVID-19 vaccines encoding SARS-CoV-  
87 2 trimeric spike protein has demonstrated the robust nature of the mRNA vaccine platform ([Baden](#)  
88 [et al., 2020](#); [Buschmann et al., 2021](#); [Sahin et al., 2020](#)). In addition to success with clinically-  
89 approved COVID-19 spike trimer vaccines, pre-clinical success has been demonstrated with  
90 nucleoside-modified mRNA encapsulated in lipid nanoparticles (mRNA-LNP) expression of Zika  
91 prM-E ([Pardi et al., 2017](#)), influenza hemagglutinin ([Pardi et al., 2018a](#); [Pardi et al., 2018c](#)), and  
92 HIV-1 envelope (Env) in gp120 monomeric or gp140 trimeric forms ([Mu et al., 2021](#); [Pardi et al.,](#)  
93 [2018a](#); [Saunders et al., 2021](#)). However, recent studies have shown that protein trimer multimers  
94 presented on a nanoparticle (NP) scaffold may be advantageous as immunogens, particularly for  
95 engaging B cell receptors (BCRs) of HIV-1 broadly neutralizing antibody (bnAb) B cell precursors  
96 that are rare or have low affinity ([Abbott et al., 2018](#); [Havenar-Daughton et al., 2018](#); [Kato et al.,](#)  
97 [2020](#); [Saunders et al., 2019](#); [Tokatlian et al., 2019](#)).

98 HIV-1 bnAbs may be disfavored by the immune system due to their unusual characteristics of  
99 long heavy-chain complementarity-determining region 3 (HCDR3) loops and polyreactivity or  
100 autoreactivity that predispose bnAbs to immune tolerance control ([Havenar-Daughton et al., 2018](#);  
101 [Haynes et al., 2019](#); [Haynes et al., 2005](#); [Haynes et al., 2012](#); [Haynes et al., 2016](#); [Huang et al.,](#)  
102 [2020](#); [Saunders et al., 2019](#); [Steichen et al., 2019](#); [Zhang et al., 2016](#)). Thus, the biology of HIV-  
103 1 bnAbs has necessitated a strategy whereby the unmutated common ancestor (UCA) or germline  
104 (GL) precursor of bnAb B cell lineages is targeted with priming immunogens to expand the bnAb  
105 precursor pool ([Haynes et al., 2019](#); [Haynes et al., 2012](#); [Jardine et al., 2013](#); [McGuire et al.,](#)  
106 [2013](#)). Following the priming immunization, Env immunogens designed to select for key antibody  
107 mutations can be administered in a specific order to guide antibody affinity maturation towards  
108 bnAb breadth and potency ([Bonsignori et al., 2017](#); [Bonsignori et al., 2016](#); [Havenar-Daughton et](#)  
109 [al., 2018](#); [Haynes et al., 2019](#); [Haynes et al., 2012](#); [Haynes et al., 2016](#); [Huang et al., 2020](#);  
110 [Saunders et al., 2019](#); [Steichen et al., 2019](#); [Zhang et al., 2016](#)). However, guiding bnAb

111 development is difficult because HIV-1 bnAbs are enriched in improbable functional somatic  
112 mutations that are required for neutralization potency and breadth ([Bonsignori et al., 2017](#); [Wiehe](#)  
113 [et al., 2018](#)). Rare somatic mutations are due to the number of nucleotide changes needed for  
114 the amino acid substitution or the lack of targeting by the somatic mutation enzyme activation-  
115 induced cytidine deaminase (AID). To promote bnAb development, Envs will need to engage  
116 those B cell receptors that have accumulated functional improbable mutations, thereby selecting  
117 intermediate bnAb B cell lineage members to proliferate and evolve further ([Bonsignori et al.,](#)  
118 [2017](#); [Haynes et al., 2012](#); [Wiehe et al., 2018](#)). Whereas bnAbs arise in ~50% of HIV-1 infected  
119 individuals ([Hraber et al., 2014](#)), to date, potent and durable bnAbs have not been induced in  
120 humans by vaccination. Together, these traits and roadblocks conspire to impede the easy  
121 induction of HIV-1 bnAbs.

122 HIV-1 Env is metastable and can adopt open and closed conformations ([Tran et al., 2012](#);  
123 [Ward and Wilson, 2017](#)). Also, Env can be triggered by its cellular receptor, CD4, to open. The  
124 Env open conformation exposes non-neutralizing antibody (nnAb) epitopes that can create  
125 competition for Env antigen between nnAb and bnAb precursors ([Havenar-Daughton et al., 2017](#);  
126 [Lee et al., 2021](#); [McGuire et al., 2014](#)). To address the problem of Env trimers opening and the  
127 exposure of non-neutralizing epitopes, multiple strategies have been designed to stabilize Env  
128 trimers in native-like conformations ([de Taeye et al., 2015](#); [Guenaga et al., 2015](#); [Henderson et](#)  
129 [al., 2020](#); [Kong et al., 2016](#)). We hypothesized that the inclusion of optimal stabilizing mutations  
130 will be critical for modified mRNAs to express antigenic and immunogenic Envs, since delivering  
131 immunogens directly as mRNA-LNP does not allow for immunogen purification. However,  
132 whether stabilizing mutations for modified mRNA expression of complex multimers will result in  
133 desired antigenicity and immunogenicity of trimer multimer NPs is not known.

134 We have previously demonstrated that a protein Env trimer designed with glycosylation sites  
135 eliminated in the first variable region (V1) of an autologous Env from an HIV-1 infected subject,  
136 CH848 (CH848 N133D N138T, CH848 10.17DT), conjugated to a ferritin nanoparticle was

137 capable of initiating a V3-glycan bnAb lineage and selecting for key improbable mutations in  
138 immunized bnAb UCA heavy and light chain variable regions ( $V_H + V_L$ ) knock-in (KI) mice  
139 ([Saunders et al., 2019](#)).

140 Here, we determined stabilization mutations in the CH848 10.17DT immunogen for formulation  
141 as mRNA-LNP. We demonstrate the modes of Env stabilization such that modified mRNA Env  
142 expression results in preferential binding to bnAbs of stabilized HIV-1 Envs in the forms of  
143 transmembrane gp160s, soluble gp140 SOSIP trimers, or gp140 SOSIP trimers on the surface of  
144 ferritin NPs encoded as a single-chain fusion gene mRNA (trimer-ferritin NPs). Moreover, we  
145 demonstrate that immunization of bnAb UCA  $V_H + V_L$  KI mice with mRNA-LNP encoding CH848  
146 10.17DT gp160s or trimer-ferritin NPs initiate a V3-glycan bnAb B cell lineage, select for bnAb  
147 lineage B cells with BCRs bearing functional improbable mutations and induce high serum titers  
148 of tier 2 V3-glycan bnAb N332-dependent autologous neutralizing antibodies. Monoclonal  
149 antibodies (mAbs) from CH848 10.17DT trimer-ferritin NP mRNA-LNP vaccinated bnAb UCA  $V_H$   
150 +  $V_L$  KI mice acquired functional bnAb lineage improbable mutations and neutralized heterologous  
151 HIV-1 isolates. Thus, the modified mRNA-LNP vaccine platform can be used to encode complex  
152 scaffolded HIV-1 trimer multimer immunogens and initiate HIV-1 bnAb maturation.

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## 154 RESULTS

### 155 Stabilization strategies for modified mRNA-encoded CH848 10.17DT Envs

156 Strategies have been proposed either to stabilize the Env trimer protein in the prefusion closed  
157 conformation or to prevent CD4-triggered structural rearrangements ([de Taeye et al., 2015](#);  
158 [Guenaga et al., 2015](#); [Henderson et al., 2020](#); [Kong et al., 2016](#); [Zhang et al., 2018](#)). We studied  
159 nine stabilization designs in CH848 10.17DT Env for expression as modified mRNAs (**Table S1**).  
160 The amino acid positions of these mutations are mapped onto the structure of CH848 10.17DT  
161 Env SOSIP trimer in **Figure 1A**.

162 The DS mutations (201C-433C) introduce a disulfide bond in the closed Env trimer and  
163 prevents CD4-triggered exposure of the CCR5 co-receptor binding site and the V3 loop ([Kwon et](#)  
164 [al., 2015](#)). The F14 mutations (68I, 204V, 208L, 255L) are designed based on a structure of  
165 BG505 SOSIP trimer complexed with BMS-626529, a small molecule that blocks soluble CD4  
166 (sCD4)-induced Env rearrangements ([Pancera et al., 2017](#)) and stabilize the SOSIP trimer by  
167 decoupling the allosteric conformational changes triggered by CD4 binding ([Henderson et al.,](#)  
168 [2020](#)). Vt8 mutations (203M, 300L, 302L, 320M, 422M) stabilize the V3 loop in the prefusion,  
169 V1/V2-coupled state ([Henderson et al., 2020](#)). The 113C-429GCG (113C-429C, 428G, 430G)  
170 and 113C-431GCG (113C-431C, 430G, 432G) mutations link the Env gp120 subunit inner and  
171 outer domains through a neo-disulfide bond, resulting in prefusion stabilized Env trimer with  
172 impaired CD4 binding ([Zhang et al., 2018](#)). For soluble gp140 trimer stabilization, we also tested  
173 SOSIPv4.1, v5.2.8 and uncleaved prefusion-optimized (UFO) mutations. Mutations in v4.1 (501C-  
174 605C, 559P, R6, ΔMPER, 535M, 543N/Q, 316W, 64K) introduce hydrophobic amino acids to  
175 disfavor solvent exposure of the V3 loop and modify gp41 in the SOSIP.664 trimer, which improve  
176 trimer formation and thermostability and decrease V3 loop exposure ([de Taeve et al., 2015](#)). The  
177 UFO design replaces the bend between alpha helices in HR1 with a computationally designed  
178 linker and aims to minimize the metastability of HIV-1 gp140 trimer ([Kong et al., 2016](#)). Mutations  
179 in the v5.2.8 design (v4.1, 66R, 73C-561C, 165L, 432Q, 429R, 65K, 106T, 49E, 47D, 500R) are  
180 designed based upon v4.1 and combine an additional disulfide bond and eight trimer-derived  
181 mutations that stabilize BG505 SOSIP trimers ([Guenaga et al., 2015](#)). Both v4.1 and v5.2.8  
182 include an improved hexa-arginine furin cleavage site R6 ([Binley et al., 2002](#)).

183

#### 184 **Antigenicity of modified mRNA-encoded CH848 10.17DT gp160s with stabilizing mutations**

185 We first designed modified mRNAs with stabilizing mutations encoding CH848 10.17DT Envs  
186 as transmembrane gp160s (**Table S1**) and tested their expression and antigenicity by transient  
187 transfection in Freestyle 293-F cells. All modified mRNA constructs expressed well and showed

188 robust V3-glycan bnAb binding (**Figures 1B and S1**). In particular, CH848 10.17DT F14, CH848  
189 10.17DT 113C-429GCG, and CH848 10.17DT 113C-431GCG gp160s exhibited binding reactivity  
190 to mature V3-glycan bnAb PGT125 and the DH270 UCA equal to that of CH848 10.17DT gp160  
191 without stabilizing mutations (**Figures 1B and S1B**). The DS, Vt8, and F14/Vt8 mutations  
192 decreased DH270 UCA binding to CH848 10.17DT gp160s (**Figure S1B**). All CH848 10.17DT  
193 gp160s showed low binding to V2-glycan bnAbs PG9 and CH01 due to lack of a lysine at position  
194 169 (K169) in the CH848 Env ([McLellan et al., 2011](#)). Thus, modified mRNA-encoded CH848  
195 10.17DT F14, CH848 10.17DT 113C-429GCG, and CH848 10.17DT 113C-431GCG gp160s  
196 showed V3-glycan UCA antibody binding that is necessary for CH848 10.17DT germline targeting.

197 To evaluate potential expression of CD4 induced (CD4i) non-neutralizing Env epitopes, we  
198 examined the susceptibility of each Env gp160 to CD4 triggering in transfected 293-F cells.  
199 Engineered (e) CD4-Ig ([Fellinger et al., 2019](#)) bound to CH848 10.17DT gp160 lacking stabilizing  
200 mutations in a dose-dependent manner, but binding of eCD4-Ig to CH848 10.17DT F14 and  
201 CH848 10.17DT 113C-429GCG gp160s was minimal (**Figure 1C**). Next, we assessed whether  
202 F14 or 113C-429GCG mutations could stabilize CH848 10.17DT gp160s in prefusion  
203 conformations and prevent the V3 loop or CCR5 co-receptor binding site exposure. Modified  
204 mRNA-transfected 293-F cells were either untreated or treated with 20 µg/ml of sCD4, eCD4-Ig  
205 ([Fellinger et al., 2019](#)) or CD4-IgG2 ([Allaway et al., 1995](#)), and Env conformation was determined  
206 by binding of CCR5 co-receptor binding site nnAb, 17b or distal V3 loop nnAb, 19b. In the absence  
207 of CD4 treatment, Env gp160s lacked binding to mAbs 17b and 19b (**Figures 1D and S1C**). After  
208 treatment with sCD4, eCD4-Ig, or CD4-IgG2, CH848 10.17DT gp160 without stabilizing mutations  
209 exhibited increased binding to both nnAbs 17b and 19b (**Figures 1D and S1C**). In contrast,  
210 stabilizing the Env gp160s with F14 or 113C-429GCG mutations completely prevented CD4-  
211 induced exposure of 17b and 19b epitopes (**Figures 1D and S1C**). Additionally, anti-gp41 nnAb  
212 7B2 against the immunodominant epitope of gp41 ([Pincus et al., 2003](#)) showed low binding to  
213 modified mRNA-expressed CH848 10.17DT Env gp160s, confirming low exposure of this gp41



214 epitope (**Figure S1C**). Thus, CH848 10.17DT gp160s with F14 and 113C-429GCG mutations  
215 were stabilized such that they preferentially bound to bnAbs versus nnAbs and non-neutralizing  
216 epitope exposure after CD4 triggering was minimal.

217

### 218 **CH848 10.17DT gp160 mRNA-LNP elicited autologous tier 2 neutralizing antibodies in vivo**

219 Based on stability and desired antigenicity, we selected CH848 10.17DT F14 and CH848  
220 10.17DT 113C-429GCG gp160s to test their immunogenicity in heterozygous V3-glycan bnAb  
221 DH270 UCA heavy and light chains ( $V_H^{+/+}$ ,  $V_L^{+/+}$ ) knock-in (DH270 UCA KI) mice ([Saunders et al.,](#)  
222 [2019](#)). Modified mRNAs encoding CH848 10.17DT F14 and CH848 10.17DT 113C-429GCG  
223 gp160s were encapsulated in ionizable LNP for immunization (**Figure 2A**). All mice immunized  
224 with CH848 10.17DT F14 or CH848 10.17DT 113C-429GCG gp160 mRNA-LNP developed  
225 serum binding IgGs to CH848 10.17DT trimer and gp120 monomer, and 3 CH848 10.17DT F14-  
226 and 4 113C-429GCG gp160 mRNA-LNP-vaccinated mice had IgGs binding to CH848 V3 peptide  
227 (**Figures 2B, S2A, and S2B**). Serum binding IgG titers to CH848 10.17DT trimer, gp120 monomer,  
228 or V3 peptide were not significantly different between CH848 10.17DT F14- and CH848 10.17DT  
229 113C-429GCG-vaccinated groups one week after the third immunization (week 5) (**Figure 2B**,  $p >$   
230 0.05, Exact Wilcoxon Mann-Whitney U test), and serum binding to V3 peptide suggested  
231 exposure of the V3 loop *in vivo*. Importantly, we observed low to non-detectable levels of binding  
232 to gp41 in both groups of mice suggesting the gp41 was not exposed upon expression in vivo  
233 (**Figure S2D**). Both groups of mRNA-LNP immunizations induced mouse serum binding  
234 antibodies to CH848 10.17DT, CH848 10.17DT F14 and CH848 10.17DT 113C-429GCG gp160s  
235 on the surface of transfected 293-F cells (**Figure S2E**).

236 Next, we asked whether CH848 10.17DT F14 and CH848 10.17DT 113C-429GCG gp160  
237 mRNA-LNP immunizations in DH270 UCA KI mice elicited serum neutralizing antibodies.  
238 Neutralizing antibody titers one week after the third immunization (week 5) were assessed by the  
239 titration of sera needed to inhibit pseudovirus replication by 50% (ID50) in TMZ-bl reporter cells

240 with a panel of 7 pseudotyped HIV-1 strains. As shown in **Figures 2C and S2F**, CH848 10.17DT  
241 F14 and CH848 10.17DT 113C-429GCG gp160 mRNA-LNP elicited autologous tier 2 (difficult-  
242 to-neutralize) ([Mascola et al., 2005](#)) neutralizing antibodies against CH848 10.17DT pseudovirus,  
243 with geometric mean titers (GMT) of ID50 at 13,175 and 10,820, respectively. Lower titers of  
244 neutralizing antibodies against CH848 10.17 virus with the V1 glycans restored (CH848 10.17)  
245 were induced that were N332 dependent, demonstrating targeting of the bnAb Env V3-glycan  
246 binding site. Moreover, comparable neutralization titers were observed against the CH848  
247 10.17DT with mutations D230N H289N P291S designed to add glycans to occlude strain-specific,  
248 immunogenic regions on the Env, indicating that most neutralizing antibodies elicited by  
249 vaccination were not targeted to these glycan-bare regions (**Figures 2C and S2F**).

250

### 251 **CH848 10.17DT gp160 mRNA-LNP selected for key DH270 bnAb mutations and elicited** 252 **germinal center responses**

253 HIV-1 bnAbs are enriched in improbable functional somatic mutations in “cold-spots” of AID  
254 enzyme activity ([Bonsignori et al., 2017](#); [Wiehe et al., 2018](#)). Splenocytes from one week after  
255 the third immunization (week 5) were subjected to next-generation sequencing (NGS) analysis.  
256 Both CH848 10.17DT F14 and CH848 10.17DT 113C-429GCG gp160 mRNA-LNP selected the  
257 critical improbable G57R mutation in the DH270 UCA V<sub>H</sub> KI gene that is necessary for the V3-  
258 glycan bnAb B cell lineage to acquire heterologous neutralization breadth ([Bonsignori et al., 2017](#);  
259 [Wiehe et al., 2018](#)), with the medians of mutation frequency at 5.4% and 3.4%, respectively  
260 (**Figure 2D**). The antibodies also acquired a second key improbable V<sub>H</sub> R98T mutation (**Figure**  
261 **2D**). Frequencies of the improbable V<sub>H</sub> G57R and the R98T mutations were comparable to those  
262 in a group of DH270 UCA KI mice immunized with Sortase ligated CH848 10.17DT Env ferritin  
263 NP protein (**Figure 2D**, P > 0.05). Thus, in DH270 UCA KI mice, CH848 10.17DT gp160 mRNA-  
264 LNP were immunogenic, induced potent N332-dependent autologous tier 2 neutralizing  
265 antibodies, and selected DH270 antibodies that acquired improbable mutations required for

266 acquisition of heterologous HIV-1 neutralization ([Bonsignori et al., 2017](#); [Saunders et al., 2019](#);  
267 [Wiehe et al., 2018](#)).

268 To examine GC responses after CH848 10.17DT gp160 mRNA-LNP immunizations in DH270  
269 UCA KI mice, splenocytes at week 5 were phenotyped for GC responses by flow cytometry using  
270 fluorophore-labeled CH848 10.17DT SOSIP trimer tetramers to detect CH848 10.17DT antigen-  
271 specific B cells (**Figure S3**). Both CH848 10.17DT F14 and CH848 10.17DT 113C-429GCG  
272 gp160 mRNA-LNP elicited CH848 10.17DT-specific GC B cells and memory B cells (**Figure 2E**).  
273 The average frequencies of CH848 10.17DT-specific GC B cells among total GC B cell population  
274 was 1.42% in CH848 10.17DT F14 gp160 mRNA-LNP group and 0.95% in CH848 10.17DT 113C-  
275 429GCG mRNA-LNP group. CH848 10.17DT F14 gp160 mRNA-LNP vaccinated group had  
276 higher frequencies of CH848 10.17DT-specific memory B cells among total memory B cells  
277 compared with CH848 10.17DT 113C-429GCG gp160 mRNA-LNP vaccinated group (mean at  
278 13.14% versus 3.88%,  $P < 0.01$ , Exact Wilcoxon Mann-Whitney U test). Additionally, CH848  
279 10.17DT gp160 mRNA-LNP elicited Tfh cell and GC Tfh cell responses in spleens in both groups  
280 (**Figures 2F and S3**).

281

## 282 **Antigenicity of modified mRNA-encoded CH848 10.17DT SOSIP trimers with stabilizing** 283 **mutations**

284 Next, antigenicity and stability of modified mRNA-encoded CH848 10.17DT SOSIP trimers  
285 with stabilizing mutations were evaluated (**Figure 1A and Table S1**). The CH848 10.17DT SOSIP  
286 trimers were chimeric with BG505 gp41 domain combined with the CH848 gp120 domain, upon  
287 which stabilizing mutations were added ([Saunders et al., 2019](#)). The antigenicity of modified  
288 mRNA-expressed *Galanthus nivalis* lectin (GNL)-purified CH848 10.17DT SOSIP trimers was  
289 measured by enzyme-linked immunosorbent assay (ELISA) using a panel of bnAbs and nnAbs.  
290 Each stabilized construct encoded by modified mRNA efficiently bound to the V3-glycan bnAbs  
291 2G12, PGT125 and PGT128 and the DH270 lineage Abs DH270 UCA, DH270 IA4 and DH270.1

292 **(Figure 3A)**. In particular, modified mRNA-encoded CH848 10.17DT SOSIP trimers with the DS  
293 mutations displayed greater binding reactivity to bnAbs, including DH270 lineage antibodies  
294 (DH270 UCA, DH270 IA4, and DH270.1) and cleaved trimer-specific gp41-gp120 interface bnAb  
295 PGT151, compared with other stabilizing mutations. Consistent with our observations with CH848  
296 10.17DT gp160s, the Vt8 and F14/Vt8 mutations decreased DH270 UCA binding to CH848  
297 10.17DT SOSIP trimers. CH848 10.17DT Vt8 and F14/Vt8 SOSIP trimers also displayed lower  
298 binding to trimer-specific bnAb PGT151 compared to CH848 10.17DT SOSIPv4.1, CH848  
299 10.17DT DS, and CH848 10.17DT F14 SOSIP trimers, suggesting less native-like conformations  
300 of Envs with these latter mutations. Little to non-detectable binding to bnAbs was observed with  
301 v5.2.8 and UFO mutations combined (v5.2.8 + UFO).

302 All stabilized constructs tested, including CH848 10.17DT DS SOSIP trimers, presented low  
303 to non-detectable levels of binding to most nnAbs, except for CH848 10.17DT SOSIPv5.2.8 that  
304 displayed about 2-fold or higher binding to nnAbs 19b and F105, compared to other stabilized  
305 Envs tested **(Figure 3A)**.

306 We assessed whether modified mRNA-expressed CH848 10.17DT SOSIP trimers with  
307 stabilizing mutations are resistant to CD4-induced opening by surface plasmon resonance (SPR).  
308 sCD4 treatment of modified mRNA-expressed non-stabilized CH848 10.17DT SOSIPv4.1 trimers  
309 increased binding of nnAb 17b **(Figure 3B)**. In contrast, CH848 10.17DT DS, CH848 10.17DT  
310 F14, and CH848 10.17DT F14/Vt8 did not show binding to 17b with or without sCD4 treatment.  
311 Although CH848 10.17DT Vt8, CH848 10.17DT SOSIPv5.2.8, and CH848 10.17DT  
312 SOSIPv5.2.8+UFO trimers exhibited increased binding to 17b after sCD4 treatment, the binding  
313 was at a lower response level compared to CH848 10.17DT SOSIPv4.1. Similar trends were  
314 observed for 19b binding. An increase in 19b binding was observed with CH848 10.17DT  
315 SOSIPv4.1 trimer, while other constructs showed low levels of binding even after sCD4 triggering  
316 **(Figure 3B)**. Thus, CH848 10.17DT DS when expressed by modified mRNA showed preferential  
317 binding to bnAbs with minimal exposure of non-neutralizing epitopes after CD4 treatment.

318 We next used size exclusion ultra-performance liquid chromatography (SE-UPLC) to define  
319 the folding of modified mRNA-encoded CH848 10.17DT SOSIP trimers. The analytical SE-UPLC  
320 profile of PGT151-purified CH848 10.17DT DS SOSIP trimer indicated that a well-folded CH848  
321 10.17DT SOSIP trimer was separated and eluted from the column as shown in **Figure 4A**. GNL-  
322 purified modified mRNA-expressed CH848 10.17 DT SOSIPv4.1 and CH848 10.17DT DS SOSIP  
323 trimer samples showed a dominant peak of trimer that was 62% and 65% of the total peak,  
324 respectively (**Figures 4B and 4C**). As shown in **Figure 4D**, negative stain electron microscopy  
325 (NSEM) analysis of CH848 10.17DT DS trimer confirmed the expression of well-folded SOSIP  
326 trimers from modified mRNA-transfected 293-F supernatant. In summary, DS mutation was the  
327 optimal stabilizing mutation strategy for CH848 10.17DT Env SOSIP trimers.

328

### 329 **Antigenicity of CH848 10.17DT SOSIP trimer-ferritin NPs with stabilizing mutations** 330 **encoded by modified mRNAs**

331 We recently demonstrated that sortase A-ligated CH848 10.17DT Env trimer ferritin NPs were  
332 potent priming immunogens for bnAb precursors ([Saunders et al., 2019](#)). Here we asked if SOSIP  
333 trimers with stabilizing mutations could be presented in an arrayed manner on ferritin and self-  
334 assemble into trimer-ferritin NPs when encoded by a single-chain modified mRNA. CH848  
335 10.17DT SOSIP trimer-ferritin NPs were produced by gene fusion of CH848 10.17DT SOSIP  
336 trimer gene with *Helicobacter pylori* (*H. pylori*) ferritin gene (*FtnA*) (GenBank NP\_223316) and  
337 were tested for expression, stability, and antigenicity (**Figure 5A**). We also constructed CH848  
338 10.17DT SOSIP trimer-ferritin NPs with CH848 strain-specific, immunogenic regions occluded by  
339 adding glycans (CH848 10.17DT with D230N, H289N, P291S) in addition to adding the E169K  
340 mutation, which is critical to interactions with V2-glycan bnAbs, including trimer-specific bnAb  
341 PGT145 ([Doria-Rose et al., 2012](#); [Lee et al., 2017](#); [McLellan et al., 2011](#)). This CH848 10.17DT  
342 Env trimer (CH848 10.17DT D230N, H289N, P291S, E169K) was termed “enhanced CH848  
343 10.17DT” (CH848 10.17DTe). Since the linker sequence connecting ferritin and Env protein would

344 affect the expression and assembly of NPs, we tested CH848 10.17DTe DS trimer-ferritin NPs  
345 with two different linkers, the sequences of which were GGGSGGGGSGLSK (termed “2xGS  
346 linker”) and GGGSGGGGSGGGGSGLSK (termed “3xGS linker”). We also designed another  
347 trimer-ferritin fusion construct using the *H. pylori* ferritin with a N19Q mutation, which removed a  
348 potential N-linked glycosylation site at position 19 and added a glycine and a serine to the C-  
349 terminus of the ferritin protein (hereafter termed the “VRC ferritin”) ([Kanekiyo et al., 2013](#)).

350 All CH848 10.17DT and CH848 10.17DTe trimer-ferritin NPs exhibited effective binding to V3-  
351 glycan bnAbs tested (**Figure 5B**). CH848 10.17DT DS and CH848 10.17DT 113C-429GCG  
352 trimer-ferritin NPs without the E169K mutation, as expected, displayed weak or no binding to V2-  
353 glycan bnAbs PGT145, CH01, PG9, and VRC26.25. Thus, modified mRNAs with stabilizing  
354 strategies tested were able to encode 10.17DT SOSIP trimer-ferritin NPs that were antigenic for  
355 bnAbs when expressed *in vitro*. In contrast, all CH848 10.17DT and CH848 10.17DTe trimer-  
356 ferritin NPs showed low to non-detectable binding to nnAbs (**Figure 5B**). Specifically, none of the  
357 trimer-ferritin NPs showed binding to 17b, and the binding to 19b was low, except for CH848  
358 10.17DT 113C-429GCG trimer-ferritin NP, indicative of an exposed distal V3 loop.

359 SPR analysis following sCD4 treatment showed no increased binding of nnAb 17b to CH848  
360 10.17DT and CH848 10.17DTe SOSIP trimer-ferritin NPs and low levels of binding of nnAb 19b  
361 (**Figure 5C**). Thus, we demonstrated that CH848 10.17DT trimer-ferritin NPs could be expressed  
362 with modified mRNAs and bound to bnAbs efficiently with limited binding to nnAbs when optimized  
363 stabilizing mutations were present. Additionally, the base part of Env trimer proteins has been  
364 shown to be highly immunogenic and some base binding antibodies can disassemble Env trimers  
365 into monomers and cause the exposure of nnAb epitopes ([Turner et al., 2021](#)). Thus, we tested  
366 binding of CH848 10.17DT trimer-ferritin NPs to an Env base binding antibody DH1029. Modified  
367 mRNA-expressed CH848 10.17DT DS SOSIP trimers without ferritin bound to DH1029 strongly,  
368 while no binding was observed to modified mRNA-expressed CH848 10.17DT trimer-ferritin NPs  
369 (**Figure 5D**), demonstrating that the immunodominant base of Env trimers was not accessible to

370 base binding antibody DH1029 recognition when presented as a multimeric nanoparticle on  
371 ferritin.

372 Next, we assessed if modified mRNA-expressed CH848 10.17DT trimer-ferritin protein indeed  
373 self-assembled into NPs by NSEM. We purified modified mRNA-transfected 293-F supernatants  
374 of CH848 10.17DT DS VRC and CH848 10.17DT DS 3xGS linker trimer-ferritin NPs by PGT145  
375 and demonstrated that CH848 10.17 DTe DS VRC ferritin and 3xGS linker ferritin mRNA  
376 transfection produced stabilized Env trimer-ferritin NPs (**Figures 5E and S4**, yellow circles). Few  
377 free trimers were observed (**Figure S4**, purple arrow). Host protein particles were classified into  
378 small 7-fold symmetry particles (**Figure S4**, yellow arrow) and large polygon-shaped particles  
379 (**Figure S4**, red arrow). The 7-fold symmetry particles were compatible with proteasomes ([Adams,  
380 2003](#)). Polygon-shaped particles have been observed in HIV-1 Env protein preparation by others  
381 ([He et al., 2016](#)), and are compatible with secreted Galectin-3 binding proteins (Gal-3BP) that  
382 assemble into ring-like polymers ([Muller et al., 1999](#); [Sasaki et al., 1998](#)), and were co-purified  
383 with HIV-1 Env NPs. Thus, NSEM analysis demonstrated that CH848 10.17DT trimer-ferritin  
384 fusion proteins self-assembled into well-folded NPs.

385

### 386 **CH848 10.17DT SOSIP trimer-ferritin NP mRNA-LNP induced autologous tier 2 neutralizing** 387 **antibodies**

388 To assess the immunogenicity of mRNA-LNP encoding CH848 10.17 DT trimer-ferritin NPs,  
389 we immunized DH270 UCA KI mice (**Figure 6A**). All CH848 10.17DT trimer-ferritin NP mRNA-  
390 LNP elicited serum antibody bound to CH848 10.17DT and CH848  $\Delta$ 11 gp120 proteins (**Figures**  
391 **6B, S5A, and S5B**). Interestingly, in contrast to CH848 10.17DT gp160s (**Figure 2B**), none of the  
392 trimer-ferritin NPs induced V3 loop peptide binding antibodies, suggesting that trimer-ferritin NPs  
393 had greater stabilization of the V3 loop (**Figures 6B and S5C**). To address the concern that using  
394 *H. pylori* ferritin induces antibodies that target the ferritin protein itself, we tested serum antibody  
395 binding to *H. pylori* ferritin used in our NPs and to human ferritin protein. We detected binding

396 activity to *H. pylori* ferritin but did not observe any immunized mouse serum cross-reactivity with  
397 human ferritin (**Figures S6E and S6F**). To assess whether trimer base-binding antibodies were  
398 elicited, we determined if immunized mouse serum contained antibodies that could block the  
399 trimer base-binding antibody DH1029. No blocking of DH1029 binding was observed in CH848  
400 10.17DT trimer-ferritin NP mRNA-LNP vaccinated mice, except for one mouse vaccinated with  
401 CH848 10.17DT 113C-429GCG trimer-ferritin NP mRNA-LNP (background cut-off at 20%). In  
402 contrast, sera from a control group of CH848 10.17DT DS SOSIP trimer protein vaccinated  
403 DH270 UCA KI mice showed DH1029 blocking activity after the second and third immunizations  
404 (**Figure 6C**). Thus, vaccination with CH848 10.17DT trimer-ferritin NP mRNA-LNP in DH270 UCA  
405 KI mice did not elicit trimer base-targeted antibodies whereas CH848 10.17DT DS SOSIP trimer  
406 protein did elicit trimer base off target antibodies.

407 Next, we assessed tier 2 serum neutralizing antibody titers after 3 immunizations against a  
408 panel of HIV-1 strains in the TZM-bl neutralization assay. All CH848 10.17DT SOSIP trimer-ferritin  
409 NP mRNA-LNP elicited neutralizing antibodies against autologous tier 2 virus CH848 10.17DT in  
410 an N332-dependent manner (**Figures 6D and S6G**). Comparable neutralizing titers against  
411 glycan holes-filled CH848 10.17DT virus (230N, 289N, 291S) were observed, indicating the  
412 antibody responses were not directed at glycan holes but rather were targeted to the V3-glycan  
413 bnAb site. CH848 10.17DTe DS VRC trimer-ferritin NP mRNA-LNP vaccinated mice showed  
414 higher neutralizing titers to CH848 10.17DT 230N, 289N, 291S viruses compared to CH848  
415 10.17DT DS ( $P < 0.05$ , Exact Wilcoxon Mann-Whitney U test) and CH848 10.17DT 113C-  
416 429GCG ( $P < 0.01$ , Exact Wilcoxon Mann-Whitney U test) trimer-ferritin NP mRNA-LNP  
417 vaccinated groups. The CH848 10.17DTe DS VRC trimer-ferritin NPs induced both the highest  
418 binding to CH848 10.17DT trimer and the highest level of tier 2 neutralizing antibodies to CH848  
419 10.17 DT and the glycan holes filled virus version (CH848 10.17DT D230N, H289N, P291S)  
420 (**Figure 6D**). Thus, CH848 10.17DT SOSIP trimer-ferritin NPs encoded as mRNA-LNP efficiently



421 elicited tier 2 autologous neutralizing antibodies that targeted the N332-dependent V3 glycan  
422 bnAb site.

423

424 **CH848 10.17DT SOSIP trimer-ferritin NP mRNA-LNP elicited germinal center responses**  
425 **and selected for key DH270 bnAb mutations**

426 All five CH848 10.17DT trimer-ferritin NPs selected the improbable V<sub>H</sub> G57R mutation in  
427 DH270 UCA KI gene, with the highest median of mutation frequency at 3.2% observed in CH848  
428 10.17DT 113C-429GCG trimer-ferritin NP vaccinated mice. Similarly, the R98T mutation in the  
429 DH270UCA V<sub>H</sub> KI gene was also selected in all groups by mRNA-LNP (**Figure 6E**). Thus, mRNA-  
430 LNP encoded CH848 10.17DT trimer-ferritin NP immunizations in DH270 UCA KI mice efficiently  
431 elicited key improbable and other mutations in DH270 intermediate antibodies.

432 All five CH848 10.17DT trimer-ferritin NP mRNA-LNP induced CH848 10.17DT-specific GC B  
433 cells and memory B cells in spleens (**Figure 6F**). Tfh cells and GC Tfh cells were also observed  
434 in all CH848 10.17DT SOSIP trimer-ferritin NP vaccinated mice (**Figure 6G**). No significant  
435 difference was observed among 5 immunization groups ( $P < 0.05$ , Exact Wilcoxon Mann-Whitney  
436 U test). Interestingly, empty LNP immunizations also elicited Tfh cell responses, albeit at much  
437 lower frequencies, consistent with previous observations that mRNA-LNP may have adjuvant  
438 effects that favors Tfh cell and GC responses ([Pardi et al., 2018a](#)).

439

440 **CH848 10.17DT trimer-ferritin NP mRNA-LNP immunization induced heterologous**  
441 **neutralizing mAbs that acquired improbable mutations**

442 To further assess antibody responses elicited by CH848 10.17DT trimer-ferritin NP mRNA-  
443 LNP, we injected DH270 UCA KI mice intradermally (i.d.) or intramuscularly (i.m.) with CH848  
444 10.17DT DS trimer-ferritin NP modified mRNA-LNP for six immunizations and sorted CH848  
445 10.17DT-specific single memory B cells on 96-well plates and amplified immunoglobulin (Ig)  
446 heavy and light chain variable regions by PCR (**Figures 7A and S5A**). We cloned a total of 397

447 Ig heavy and light chain pairs, 228 (57%) pairs of which used DH270 KI genes *IGHV1-2* and  
448 *IGVL2-23*. Among these 228 DH270-like antibodies, 173 (76%) antibodies have acquired at least  
449 one amino acid mutation (**Figures 7B and S5B**). We aligned all unique VH1-2/VL2-23 Ig gene  
450 amino acid sequences with bnAb DH270.6, and found that the Ig heavy chain group accumulated  
451 a total of 14 out of 19 (74%) DH270.6 probable mutations and 4 out of 8 (50%) DH270.6  
452 improbable mutations (**Figure S7**). Similarly, the Ig light chain group accumulated 6 out of 9 (67%)  
453 DH270.6 probable mutations and 4 out of 6 (67%) improbable mutations (**Figure S8**). Specifically,  
454 5 (2%) and 20 (9%) Ig heavy chains acquired the DH270.6 bnAb G57R and the R98T improbable  
455 mutations, respectively; and 2 (1%) Ig light chains acquired the DH270.6 bnAb L48Y improbable  
456 mutation (**Figure 7C**). Binding reactivity of cloned antibodies were screened in ELISA (**Table S3**),  
457 and antibodies with heterologous HIV-1 A.Q23 Env binding were selected for further assessment.  
458 Among them, three mAbs (DH270.mo84, DH270.mo85, and DH270.mo86) were identified that  
459 showed strong binding to CH848 10.17DT, CH848 10.17, and heterologous HIV-1 A.Q23 SOSIP  
460 trimers (**Figure 7D**). We then assessed neutralization of these three mAbs against a panel of 17  
461 HIV-1 isolates that the first intermediate ancestor antibody (DH270 IA4) in the DH270 lineage  
462 neutralizes ([Saunders et al., 2019](#)) (**Figure 7D**). Each of the 3 mAbs neutralized autologous tier  
463 2 CH848 viruses and heterologous 92RW020 and 6101.1 viruses with titers comparable to DH270  
464 IA4 ([Saunders et al., 2019](#)). Antibody DH270.mo84 also neutralized each of the 13 other  
465 heterologous HIV-1 isolates tested. Antibodies DH270.mo85 and DH270.mo86 neutralized 8 and  
466 10 of 13 heterologous isolates, respectively (**Figure 7D**). DH270.mo84 encoded the V<sub>H</sub> G57R  
467 improbable mutation, DH270.mo86 encoded the V<sub>H</sub> R98T improbable mutation, while  
468 DH270.mo85 had both the G57R and R98T improbable mutations. Additionally, DH270.mo86  
469 acquired V<sub>L</sub> S27Y and V<sub>L</sub> S57N improbable mutations (**Figure 7E**). Thus, CH848 10.17DT DS  
470 trimer-ferritin NP mRNA-LNP immunization induced heterologous tier 2 neutralizing DH270  
471 antibodies with improbable mutations.

472

473 **DISCUSSION**

474 Nucleoside-modified mRNAs in lipid nanoparticles (mRNA-LNP) represent an exciting new  
475 platform for viral vaccine development for experimental HIV-1 vaccines ([Baden et al., 2020](#); [Mu](#)  
476 [et al., 2021](#); [Pardi et al., 2018b](#); [Polack et al., 2020](#)). A major question is if mRNA designs should  
477 incorporate stabilizing mutations in trimers or NPs to optimize immunogen expression and stability  
478 since the protein products of mRNAs cannot be purified after mRNA-LNP injection *in vivo*. In this  
479 study, we evaluated mutations that stabilize modified mRNA-encoded Envs expressed as  
480 transmembrane gp160s, soluble SOSIP trimers, or single-gene mRNA trimer-ferritin NPs. For  
481 mRNAs encoding the V3-glycan germline targeting CH848 10.17DT Env ([Saunders et al., 2019](#)),  
482 we showed that F14 and 113C-429GCG mutations optimally stabilized transmembrane gp160s,  
483 DS mutation best stabilized SOSIP trimers, and were also able to optimally stabilize trimer-ferritin  
484 NPs expressed from modified mRNA.

485 Since we have previously demonstrated that immunization with Env ferritin NPs is superior to  
486 soluble trimers alone ([Saunders et al., 2019](#)), we determined immunogenicity of mRNA-LNP  
487 encoding transmembrane Env gp160 and trimer-ferritin NPs for their ability to expand UCAs of  
488 V3-glycan DH270 bnAb B cell lineage and to select for desired, functional bnAb mutations.  
489 mRNA-LNP encoding both transmembrane gp160s and soluble trimer-ferritin NPs induced high  
490 titers of autologous bnAb-targeted tier 2 neutralizing antibodies with groups of mutations present  
491 in bnAb intermediate antibodies ([Bonsignori et al., 2017](#); [Saunders et al., 2019](#)). Additionally,  
492 mAbs with heterologous neutralizing activities and functional improbable mutations were isolated  
493 after trimer-ferritin NP mRNA-LNP vaccination. Moreover, we observed accumulation of mature  
494 bnAb DH270.6 mutations in vaccine-induced mAbs, although mutations are distributed across all  
495 isolated mAbs. This observation suggests that mRNA-LNP immunogens are selecting for key  
496 bnAb mutations, so the next goal is to have mutations concatenated on one or two mAbs. To  
497 achieve this, prolonged GC responses or enhanced recruitment of memory B cells back into GCs

498 will likely need to be induced by vaccination to allow bnAb lineage B cell BCRs to acquire more  
499 mutations.

500 Eliciting bnAbs by vaccination has not been successful. However, studies in HIV-1-infected  
501 individuals have demonstrated that those who make bnAbs have higher levels of T follicular helper  
502 (Tfh) cells ([Locci et al., 2013](#); [Moody et al., 2016](#)), NK cell dysfunction ([Bradley et al., 2018](#)),  
503 defects in T regulatory cells (Treg) ([Moody et al., 2016](#)), and B cell repertoires containing longer  
504 HCDR3-bearing B cells and autoreactive B cells that normally are deleted ([Roskin et al., 2020](#)).  
505 Nucleoside-modified mRNA-LNP vaccines selectively induce high levels of Tfh cells and minimize  
506 induction of Treg cells ([Pardi et al., 2018a](#)), and thus will be a key platform for bnAb lineage  
507 initiation and selection of B cells with improbable functional mutations that facilitate bnAb  
508 maturation. The CH848 10.17DTe DS trimer-ferritin NP is currently in good manufacturing  
509 practice (GMP) production to investigate the priming of such lineages in humans both as mRNA-  
510 LNP or recombinant protein.

511

512 In summary, we have demonstrated that single-chain mRNAs can be designed to encode  
513 complex molecules such as HIV-1 Env trimer-ferritin NP and that these immunogens are capable  
514 of selecting for difficult-to-elicite improbable mutations critical for broad tier 2 virus neutralization.  
515 The complex biology of HIV-1 bnAbs necessitates a vaccine strategy that utilizes a series of  
516 sequentially administered Env immunogens that initially expand bnAb precursors and then select  
517 for improbable mutations ([Haynes et al., 2019](#); [Haynes et al., 2012](#); [Saunders et al., 2019](#); [Wiehe  
518 et al., 2018](#)). Manufacturing of complex nanoparticle protein immunogens in large-scale is faced  
519 with significant practical and funding challenges. The use of mRNA-LNP has the possibility of  
520 making such a complex immunization regimen both logistically achievable and potentially cost-  
521 effective.

522

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534

#### 535 **AUTHOR CONTRIBUTIONS**

536 Conceptualization, Z.M., K.O.S., D.W. and B.F.H.; Methodology, Z.M., R.P., K.O.S., and  
537 B.F.H.; Software, S.V. and K.J.W., Investigation Z.M., K.J.W., K.O.S., R.H., D.W.C., R.P., D.M.,  
538 K.M., R.J.E., A.N., X.L., S.X., M.B., D.M., Q.H., S.V., T.E., M.A., W.B.W., N.P., D.W., B.F.H.;  
539 Statistical analysis, Y.W., W.R.; Writing, Z.M., K.O.S., K.J.W., and B.F.H.; Funding Acquisition,  
540 B.F.H.; Resources, Y.T., C.B., N.P., D.W.; B.F.H. Supervision, B.F.H.

541

#### 542 **DECLARATION OF INTERESTS**

543 B.F.H., K.O.S., and K.W. have patent applications on some of the concepts and immunogens  
544 discussed in this paper.

545

#### 546 **FIGURE LEGENDS**

547 **Figure 1. Antigenicity of modified mRNA-encoded CH848 10.17DT gp160 with stabilizing**  
548 **mutations.**

549 **(A)** Amino acid positions of stabilizing mutations tested in this study mapped onto structure of  
550 CH848 10.17DT SOSIP trimer (PDB ID: 6UM5). One protomer is shown in rainbow color and the  
551 other two protomers are shown in grey. Amino acid mutations in each stabilizing strategy are  
552 listed in boxes. Black fonts outside of boxes are mutations in v4.1, and blue fonts are mutations  
553 in v5.2.8, in addition to v4.1 mutations. Residue 561C in v5.2.8 or redesigned HR1 region in UFO  
554 mutation is not shown due to lack of HR1 region in this structure.

555 **(B)** Antigenicity of modified mRNA-expressed CH848 10.17DT (purple), CH848 10.17DT F14  
556 (blue), and CH848 10.17DT 113C-429GCG (orange) transmembrane gp160s measured by  
557 binding of V3-glycan bnAb PGT125 and the unmutated common ancestor of DH270 bnAb (DH270  
558 UCA). Data were shown as means  $\pm$  standard error of mean (SEM) of PE+ cell percentage among  
559 live cell population from three independent experiments.

560 **(C)** eCD4-Ig binding reactivity to modified mRNA-expressed CH848 10.17DT, CH848 10.17DT  
561 F14, and CH848 10.17DT 113C-429GCG gp160s measured by flow cytometry. Data shown are  
562 means of mean fluorescent intensity (MFI) from three independent experiments. Error bars, mean  
563  $\pm$  SEM.

564 **(D)** Binding reactivity of non-neutralizing antibodies (nnAbs) 17b (left) and 19b (right) to modified  
565 mRNA-expressed CH848 10.17DT, CH848 10.17DT F14, and CH848 10.17DT 113C-429GCG  
566 gp160s with or without treatment with sCD4, eCD4-Ig, and CD4-IgG2. Binding of nnAbs was  
567 shown as means  $\pm$  SEM of PE+ cell percentage among live cell population from three independent  
568 experiments.

569 **See also Figure S1 and Table S1.**

570

571 **Figure 2. Immunogenicity of CH848 10.17DT gp160 mRNA-LNP in mice.**

572 **(A)** Immunization schema in DH270 UCA dKI mice with CH848 10.17DT F14 gp160 mRNA-LNP  
573 (blue) and CH848 10.17DT 113C-429GCG gp160 mRNA-LNP (orange).

574 **(B)** Week 5 serum antibody binding to CH848 10.17DT SOSIP trimer, CH848 10.17  $\Delta$ 11 gp120,  
575 and CH848 V3 loop peptide measured by ELISA. Data shown are log transformed area-under-  
576 curve (logAUC). Each dot represents an individual mouse (N = 6 each group). No significant  
577 statistical difference was observed between two groups ( $P > 0.05$ ).

578 **(C)** Week 5 serum neutralizing antibody titers measured in TZM-bl reporting cells with a panel of  
579 autologous and heterologous tier 2 HIV-1 pseudoviruses. Murine leukemia virus (MuLV) was used  
580 as negative control. Neutralization titers are reported as the serum dilution that inhibit 50% of virus  
581 replication (ID50). Each dot signifies an individual mouse (N = 6 each group). Horizontal bar  
582 indicates geometric mean titer (GMT) of ID50 in each group. No significant statistical difference  
583 was observed between two groups ( $P > 0.05$ ).

584 **(D)** Improbable G57R and R98T mutation frequencies in DH270 V<sub>H</sub> KI gene after CH848 10.17DT  
585 gp160 mRNA-LNP immunizations. Frequencies were compared to three CH848 10.17DT Sortase  
586 ferritin NP protein immunizations and empty LNP immunizations. Each dot represents an  
587 individual mouse (N = 6 in each group). Horizontal bar: median.

588 **(E)** CH848 10.17DT gp160 mRNA-LNP vaccination induced GC B cell responses in spleen.  
589 Necropsy was performed on week 5 and splenocytes were subjected to GC responses  
590 immunophenotyping by flow cytometry. Frequencies of CH848 10.17DT SOSIP trimer-specific GC  
591 B cells among total GC B cells (left) and CH848 10.17DT SOSIP trimer-specific memory B cells  
592 among total memory B cells (right) in splenocytes of CH848 10.17DT F14 gp160 mRNA-LNP  
593 (blue) or CH848 10.17DT 113C-429GCG gp160 mRNA-LNP (orange) vaccinated DH270 UCA  
594 dKI mice. Each dot represents an individual mouse (N = 6 in each group). Horizontal bar indicates  
595 means in each group. \*\*  $P < 0.01$ .

596 **(F)** CH848 10.17DT gp160 mRNA-LNP vaccination induced GC Tfh cell responses in spleen.  
597 Frequencies of total Tfh (left) and GC Tfh cells (right) among CD4<sup>+</sup> T cells in splenocytes at week  
598 5 after CH848 10.17DT F14 gp160 mRNA-LNP or CH848 10.17DT 113C-429GCG gp160 mRNA-  
599 LNP vaccination in DH270 UCA dKI mice were assessed by flow cytometry. Each dot represents

600 an individual mouse (N = 6 in each group). Horizontal bar indicates means in each group. No  
601 significant statistical difference was observed between two groups ( $P > 0.05$ ).

602 Significance was determined using Exact Wilcoxon Mann-Whitney U test.

603 **See also Figures S2 and S3.**

604

605 **Figure 3. Antigenicity of modified mRNA-encoded CH848 10.17DT SOSIP trimers with**  
606 **stabilizing mutations.**

607 **(A)** BnAb/bnAb precursor and nnAb binding reactivity to modified mRNA-expressed CH848  
608 10.17DT SOSIP trimers with various stabilizing mutations. Antibody binding was measured by  
609 ELISA. Data shown are means of logAUC from three independent experiments.

610 **(B)** SPR sensorgrams of nnAb 17b or 19b binding to modified mRNA-expressed CH848 10.17DT  
611 SOSIP trimers with (blue) or without (red) sCD4 treatment. Antibodies 17b or 19b were  
612 immobilized onto a sensor chip. Modified mRNA-expressed GNL-purified CH848 10.17DT SOSIP  
613 trimers incubated with and without sCD4 were injected over the sensor chip surface. The protein  
614 was then allowed to dissociate for 600 seconds.

615 **See also Table S1.**

616

617 **Figure 4. Modified mRNA-expressed CH848 10.17DT DS SOSIP trimer is well-folded.**

618 **(A)** Analytical size-exclusive ultra-performance liquid chromatography (SE-UPLC) profile of  
619 CH848 10.17DT SOSIP trimer protein standard purified by bnAb PGT151. CH848 10.17DT  
620 SOSIP trimer elutes from the column at about 7 min.

621 **(B-C)** Analytical SE-UPLC profile of modified mRNA-expressed GNL-purified **(B)** CH848 10.17DT  
622 SOSIPv4.1 trimers (orange) and **(C)** CH848 10.17DT DS SOSIP trimers (orange). Black curve  
623 indicates GNL-purified material from mock transfection.

624 **(D)** Negative-stain electron microscopy (NSEM) analysis of modified mRNA-expressed GNL-  
625 purified CH848 10.17DT DS SOSIP trimers. Shown on the left is a representative NSEM



626 micrograph of CH848 10.17DT DS SOSIP trimer and on the right are 2D classification of well-  
627 folded trimers.

628

629 **Figure 5. Antigenicity of CH848 10.17DT SOSIP trimer-ferritin NPs with stabilizing**  
630 **mutations.**

631 **(A)** Design of CH848 10.17DT SOSIP trimer-ferritin fusion NPs. The CH848 10.17DT SOSIP gene  
632 is genetically fused to *Helicobacter pylori* Ferritin gene (*FtnA*) by a linker sequence. Ferritin self-  
633 assembles into a 24-mer nanoparticle, with 8 SOSIP trimers on the surface.

634 **(B)** BnAb/bnAb precursor and nnAb binding reactivity to modified mRNA-expressed CH848  
635 10.17DT SOSIP trimer-ferritin NPs with various stabilizing mutations. Antibody binding was  
636 measured by ELISA. Data shown are means of logAUC from three independent experiments.

637 **(C)** SPR sensorgrams of nnAb 17b or 19b binding to modified mRNA-expressed CH848 10.17DT  
638 SOSIP trimer-ferritin NPs with (blue) or without (red) sCD4 treatment. Antibodies 17b or 19b were  
639 immobilized onto a sensor chip. Modified mRNA-expressed GNL-purified CH848 10.17DT SOSIP  
640 trimer-ferritin NPs incubated with and without sCD4 were injected over the sensor chip surface.  
641 The protein was then allowed to dissociate for 600 seconds.

642 **(D)** Binding reactivity of Env base antibody DH1029 to modified mRNA-expressed CH848  
643 10.17DT DS SOSIP trimer (blue) or CH848 10.17DTe DS 2xGS linker (green), CH848 10.17DTe  
644 DS 3xGS linker (purple), and CH848 10.17DTe DS VRC (orange) trimer-ferritin NPs measured  
645 by ELISA. Data shown are means of absorbance at 450nm from at least two independent  
646 experiments.

647 **(E)** Representative NSEM images (right) and 2D classifications (left) of modified mRNA-  
648 expressed, PGT145-purified CH848 10.17DTe DS VRC trimer-ferritin NPs (top) and CH848  
649 10.17DTe DS 3xGS linker trimer-ferritin NPs (bottom).

650 **See also Figure S4 and Table S1.**

651

652 **Figure 6. Immunogenicity of CH848 10.17DT SOSIP trimer-ferritin NP mRNA-LNP in mice.**

653 **(A)** Immunization schema with CH848 10.17DT SOSIP trimer-ferritin NP mRNA-LNP in DH270  
654 UCA dKI mice.

655 **(B)** Serum IgG binding to CH848 10.17DT SOSIP trimer, CH848 10.17  $\Delta$ 11 gp120, and CH848  
656 V3 peptide. Each dot represents an individual mouse (N = 5 in Group 1; N = 6 in the rest of  
657 groups). \* P < 0.05, \*\* P < 0.01.

658 **(C)** DH1029 blocking by sera from mice vaccinated with CH848 10.17DT trimer-ferritin NP mRNA-  
659 LNP. CH848 10.17DT trimer protein vaccinated mice from one of our previous studies were  
660 included as a positive control (brown). Dotted horizontal line indicates background cut-off at 20%  
661 blocking.

662 **(D)** Serum neutralizing titers after CH848 10.17DT SOSIP trimer-ferritin NP mRNA-LNP  
663 immunizations measured in TZM-bl reporter cells. MuLV was used as negative control.  
664 Neutralization titers are reported as the serum dilution that inhibit 50% of virus replication (ID50).  
665 Each dot signifies an individual mouse (N = 5 in Group 1; N = 6 in the rest of groups). Horizontal  
666 bar indicates geometric mean titer (GMT) of ID50. \* P < 0.05, \*\* P < 0.01.

667 **(E)** Improbable G57R and R98T mutations frequency in DH270 V<sub>H</sub> KI gene after CH848 10.17DT  
668 SOSIP trimer-ferritin mRNA-LNP immunizations. Frequencies were compared to three CH848  
669 10.17DT Sortase ferritin NP protein immunizations and empty LNP immunizations. \* P < 0.05, \*\*  
670 P < 0.01.

671 **(F)** Frequencies of CH848 10.17DT-specific GC B cells and memory B cells in splenocytes of  
672 CH848 10.17DT SOSIP trimer-ferritin mRNA-LNP vaccinated mice. \* P < 0.05

673 **(G)** Frequency of CH848 10.17DT-specific GC Tfh cells and memory B cells in spleen of CH848  
674 10.17DT SOSIP trimer-ferritin mRNA-LNP vaccinated mice. \* P < 0.05

675 Significance was determined by Exact Wilcoxon Mann-Whitney U test, without any P value  
676 adjustment for multiple comparison.

677 **See also Figures S5 and S3.**

678

679 **Figure 7. CH848 10.17DT DS trimer-ferritin NP mRNA-LNP vaccination elicited antibodies**  
680 **that acquired improbable mutations and neutralization breadth.**

681 **(A)** Immunization schema with CH848 10.17DT DS SOSIP trimer-ferritin NP mRNA-LNP in  
682 DH270 UCA dKI mice. Necropsy was performed one week after the sixth immunization.

683 **(B)** Representative gate for CH848 10.17DT Env-specific single memory B cell sorting from  
684 DH270 UCA dKI mice splenocytes one week after the sixth vaccination with CH848 10.17DT DS  
685 trimer-ferritin NP mRNA-LNP.

686 **(C)** Summary of Ig gene recovery by PCR from single-cell sorted memory B cells. Left: A total of  
687 397 Ig heavy and light chain gene pairs were recovered. 228 (57%) of cloned Ig gene pairs used  
688 DH270 *IGVH1-2* and *IGVL2-23* genes, and thus were considered as DH270-like Abs. Ig gene  
689 pairs that used only one of DH270 heavy chain or light chain, or endogenous mouse Ig genes are  
690 categorized as “Other Ab”. Right: The number and percentage of the 228 DH270-like VH1-2/VL2-  
691 23 Abs that had acquired at least one amino acid change in heavy or light chain (N = 173, 76%).  
692 VH1-2/VL2-23 Abs without full length, clean VDJ/VJ sequences are categorized as  
693 “Undetermined”.

694 **(D)** Summary of improbable mutations in VH1-2/VL2-23 Abs. Among the 228 DH270-like VH1-  
695 2/VL2-23 Abs, 5 (2%) and 20 (9%) of them have acquired the V<sub>H</sub> G57R and the R98T improbable  
696 mutations, respectively; 2 (1%) have acquired the V<sub>L</sub> L48Y improbable mutation.

697 **(E)** Binding reactivity of three cloned mAbs DH270.mo84, DH270.mo85, and DH270.mo86 to a  
698 panel of CH848 10.17DT proteins measured by ELISA. All three antibodies bound to CH848  
699 10.17DT SOSIP trimers and CH848 10.17 SOSIP trimers, while no binding to CH848 10.17DT  
700 N332T was observed. Binding to BG505 T332N and heterologous Q23 SOSIP trimers was also  
701 observed.

702 **(F)** DH270.mo84, DH270.mo85, and DH270.mo86 neutralization activity against a panel of 17  
703 HIV-1 isolates. Data shown are antibody concentration that inhibit 50% of virus replication (IC50)  
704 in TZM-bl assay. MuLV was used as negative control.

705 **(G)** Improbable mutations in mAbs DH270.mo84, DH270.mo85, and DH270.mo86. Alignment of  
706 mAb heavy and light chain sequences with DH270 UCA sequences. Mutations are highlighted  
707 and improbable mutations are shown in red fonts.

708 **See also Figures S5-S7 and Table S3.**

709

## 710 **STAR Methods**

## 711 **LEAD CONTACT**

712 Further information and requests for resources and reagents should be directed to and will be  
713 fulfilled by the lead contact Barton F. Haynes ([barton.haynes@duke.edu](mailto:barton.haynes@duke.edu)).

714

## 715 **MATERIALS AVAILABILITY**

716 This study did not generate new unique reagents.

717

## 718 **DATA AND CODE AVAILABILITY**

719 Any additional information required to reanalyze the data reported in this paper is available from  
720 the lead contact upon request.

721

## 722 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

### 723 **Cell line**

724 Freestyle 293-F cell line (Thermo Fisher Scientific, Cat# R79007) was purchased from Thermo  
725 Fisher and cultured in Freestyle 293 Expression Medium (Thermo Fisher Scientific, Cat# 12338-  
726 026). Cells were maintained in 8% CO<sub>2</sub> at 37°C at a density between 0.3x10<sup>6</sup>/ml to 3x10<sup>6</sup>/ml.

727 Mycoplasma test was performed when a new stock vial was thawed at Duke University Cell  
728 Culture Facility.

729

### 730 **Animals and immunizations**

731 The DH270 UCA dKI mice has been previously described ([Saunders et al., 2019](#)). For CH848  
732 10.17DT gp160 mRNA-LNP immunizations, 12 DH270 UCA dKI mice were randomly split into  
733 two groups (N = 6 each group) and were immunized intramuscularly (i.m.) with 20 µg of mRNA-  
734 LNP encoding CH848 10.17DT F14 gp160 and CH848 10.17DT 113C-429GCG gp160 every two  
735 weeks for three times. For CH848 10.17DT DS trimer-ferritin NP immunizations were done  
736 similarly, except that the second and the third immunizations were only one week apart. Control  
737 group mice were injected with 20 µg of empty LNP. Bleeding was performed one week after each  
738 immunization. Necropsy was performed one week after the third immunization and blood, spleen,  
739 and lymph nodes were collected. All mice were cared for in a facility accredited by the Association  
740 for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All study  
741 protocol and all veterinarian procedures were approved by the Duke University Institutional  
742 Animal Care and Use Committee (IACUC).

743

### 744 **METHOD DETAILS**

#### 745 **Modified mRNA production**

746 Modified mRNAs were produced by *in vitro* transcription using T7 RNA polymerase  
747 (Megascript, Ambion) on linearized plasmids encoding codon-optimized CH848 10.17DT gp160s,  
748 CH848 10.17DT SOSIP trimers, or CH848 10.17DT trimer-ferritin NPs. All the HIV-1 modified  
749 mRNA constructs used in this study and their corresponding plasmids were listed in **Table S1**.  
750 One-methylpseudouridine (m<sup>1</sup>Ψ)-5'-triphosphate (TriLink, Cat# N-1081), instead of UTP was  
751 used to produce nucleoside-modified mRNAs. Modified mRNAs contain 101 nucleotide-long  
752 polyadenylation tails for optimized expression. Modified CH848 10.17DT SOSIPv4.1 trimer and

753 CH848 10.17DT SOSIPv5.2.8 trimer mRNAs were capped using ScriptCap m7G capping system  
754 and ScriptCap 2'-O-methyl-transferase kit (ScriptCap, CellScript) ([Pardi et al., 2013](#)). Capping of  
755 all other *in vitro* transcribed mRNAs was performed co-transcriptionally using the trinucleotide  
756 cap1 analog, CleanCap (TriLink, Cat# N-7413). All mRNAs were purified by cellulose purification,  
757 as described ([Baierdorfer et al., 2019](#)). All mRNAs were analyzed by agarose gel electrophoresis  
758 and were stored frozen at -20 °C.

759

### 760 **Nucleoside-modified mRNA-LNP production**

761 Nucleoside-modified mRNAs were encapsulated in LNP for mouse immunizations as  
762 previously described ([Jayaraman et al., 2012](#); [Maier et al., 2013](#)). Modified mRNAs in aqueous  
763 phase were rapidly mixed with a solution of lipids dissolved in ethanol. LNP formulation contains  
764 ionizable cationic lipid (proprietary to Acuitas)/phosphatidylcholine/cholesterol/PEG-lipid. The  
765 cationic lipid and LNP composition are described in US patent US10,221,127 ([Du, 2019](#)).

766

### 767 **Nucleoside-modified mRNA transfection in 293-F cell line**

768 293-F cells were diluted to  $0.7 \times 10^6$  cells/ml 24 h before transfection. On the next day, cells  
769 were diluted again to  $1 \times 10^6$ /ml and seeded into tissue culture plates for transfection. 3 µg of  
770 mRNAs expressing gp160s were transfected into 6 ml of cells. For soluble SOSIP trimers, 30 ml  
771 of cells were transfected with 12 µg of SOSIP-expressing mRNAs and 3 µg of Furin mRNAs.  
772 Transfection volume were doubled to 60 ml for trimer-ferritin NPs. TransIT-mRNA Transfection  
773 Kit (Mirus Cat# MIR2250) was used for mRNA transfection following the manufacturer's  
774 instructions. Transfected cells were cultured at 37°C with 8% CO<sub>2</sub> and shaking at 120 rpm for 48  
775 h (for gp160) or 72 h (for SOSIP trimers and trimer-ferritin NPs) before harvest.

776

777 **Evaluation of expression and folding of modified mRNA-expressed CH848 10.17DT gp160s,**  
778 **SOSIP trimers, and trimer-ferritin NPs**

779 The expression and folding of modified mRNA-encoded CH848 10.17DT transmembrane  
780 gp160s, Soluble SOSIP trimers, and trimer-ferritin NPs were defined as follows. For CH848  
781 10.17DT transmembrane gp160s, flow cytometry was used to measure binding of a panel of  
782 bnAbs and nnAbs. BnAb binding reactivity indicated successful expression of gp160 Envs on cell  
783 surface with desired antigenicity. Binding of nnAbs 17b and 19b measured the ability of various  
784 stabilizing mutations to keep the gp160 Envs in prefusion conformation and to decrease the  
785 exposure of non-neutralizing epitopes CCR5 binding site and distal V3 loop. Additionally, 7B2  
786 binding was used to measure the exposure of gp41.

787 For CH848 10.17DT soluble trimer, total Env forms were purified by *Galanthus nivalis* lectin  
788 (GNL) from modified mRNA-transfected 293-F cell supernatant. A panel of nnAbs and bnAbs  
789 were used in ELISA to measure the expression of non-neutralizing and neutralizing epitopes.  
790 Binding of nnAbs 17b and 19b after CD4 triggering were measured by SPR. To assess the percent  
791 of trimeric Envs in GNL-purified materials, size-exclusion ultra-performance liquid  
792 chromatography (SE-UPLC) analysis was performed with PGT151 affinity-purified CH848  
793 10.17DT SOSIP trimer as a standard. Finally, Negative-stain Electron Microscopy (NSEM)  
794 analysis was performed to confirm trimer formation in GNL-purified 293-F transfection  
795 supernatant.

796 Similar antigenicity measurement and SPR analysis was performed on GNL-purified 293-F  
797 cell supernatant transfected with modified mRNA expressing CH848 10.17DT trimer-ferritin NPs  
798 to evaluate the expression of well-folded Env trimers on the ferritin nanoparticle. Additionally,  
799 transfected 293-F supernatant were affinity purified with PGT145-conjugated beads, which  
800 exclude host glycan proteins that may be purified by GNL. PGT145-purified materials were  
801 analyzed by NSEM to confirm the assembly of CH848 10.17DT DS ferritin NPs.

802

803 *Flow cytometry*

804 Binding of bnAbs to CH848 10.17DT gp160s was performed by flow cytometry as previously  
805 described ([Henderson et al., 2020](#); [Saunders et al., 2021](#)). Briefly, modified mRNA-transfected  
806 293-F cells were harvested 48 h after transfection and were washed once with 1% BSA in PBS.  
807 Then, cells were incubated with 10 µg/ml of bnAbs in V-bottom 96-well plates for 30 min at 4 °C.  
808 Cells were then washed with 1% BSA in PBS and incubated with Goat F(ab')<sub>2</sub> Anti-Human IgG -  
809 (Fab')<sub>2</sub> (PE) (Abcam Cat# ab98606, RRID:AB\_10672217) for 30 min at 4 °C in dark. Then, cells  
810 were washed once with PBS and dead cells were stained with LIVE/DEAD Fixable Aqua Dead  
811 Cell Stain Kit (Invitrogen Cat# L34957, 1:1000 dilution in PBS) for 15 min at 4 °C in dark, then  
812 washed twice and re-suspended in 1% BSA in PBS. Flow cytometric data were acquired on a  
813 LSRII High-throughput system using FACSDIVA software (BD Biosciences) and were analyzed  
814 with FlowJo software (FlowJo). The percentage of 293-F cells that were PE positive was shown  
815 in the results.

816 Measurement of binding of nnAbs after CD4 treatment has been described previously  
817 ([Henderson et al., 2020](#)). Briefly, mRNA-transfected 293-F cells were first incubated with 20 µg/ml  
818 of soluble CD4 (sCD4), eCD4-Ig or CD4-IgG2 for 10 min at 4 °C. Cells were washed once with  
819 1% BSA in PBS and then incubated with 10 µg/ml of nnAbs 17b, 19b or 7B2 for 30 min at 4°C.  
820 Then, cells were incubated with Goat F(ab')<sub>2</sub> Anti-Human IgG - (Fab')<sub>2</sub> (PE) and dead cells were  
821 stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit. Data acquisition and analysis were  
822 the same as described above.

823

#### 824 *Galanthus nivalis* lectin purification of SOSIP trimers and trimer-ferritin NPs

825 293-F cells transfected with modified mRNAs expressing SOSIP trimers or trimer-ferritin NPs  
826 were harvested 72 h after transfection and were centrifuged for 30 min at 3000 rpm to remove  
827 cells and debris. Supernatant were first filtered using a 0.22 µm vacuum filter and were then  
828 concentrated by 50-fold using 10 kDa MWCO concentrators. Concentrated supernatant was  
829 incubated with 200 µl of agarose bound *Galanthus nivalis* lectin (GNL) (Vector Laboratories Cat#



830 AL-1243) with gentle rotation at 4 °C overnight. The next day, GNL agarose beads were washed  
831 with MES wash buffer (20 mM MES, 130 mM NaCl, 10 mM CaCl<sub>2</sub> pH 7.0) for three times, and  
832 SOSIP trimers or trimer-ferritin NPs were eluted by 500 mM Methyl alpha-D-mannopyranoside in  
833 MES wash buffer. The eluates were then dialyzed to 10 mM Tris-HCl pH8 500 mM NaCl using  
834 30kDa MWCO spin concentrators. GNL-purified SOSIP trimers or trimer-ferritin NPs were snap-  
835 frozen and stored in -80 °C.

836

### 837 *Enzyme-linked immunosorbent assay (ELISA)*

838 Binding reactivity of modified mRNA-expressed CH848 10.17DT SOSIP trimers and trimer-  
839 ferritin NPs to bnAbs and nnAbs was measured by enzyme-linked immunosorbent assay (ELISA).  
840 In brief, HIV-1 antibodies were coated onto 384-well assay plates in 0.1M Sodium bicarbonate  
841 overnight at 4 °C. GNL-purified SOSIP trimers or trimer-ferritin NPs with serial dilutions were then  
842 captured on the plates. Next, poly-serum from CH848 10.17DT-immunized rhesus macaque was  
843 incubated for 1 h at room temperature. Then, Mouse Anti-Monkey IgG-HRP (SouthernBiotech  
844 Cat# 4700-05, RRID:AB\_2796069) was incubated for 1 h at room temperature and plates were  
845 developed with SureBlue Reserve TMB 1-Component Microwell Peroxidase Substrate (Seracare  
846 Cat# 5120-0083) for 15 min and were stopped with 1% HCl solution. Absorbance at 450 nm were  
847 determined by SpectraMax Plus 384 microplate reader (Molecular Devices) and log area-under-  
848 curve (log AUC) were calculated using Prism (Graphpad) and shown in figures.

849 The base binding antibody assay was performed similarly. Briefly, base binding antibody  
850 DH1029 was coated onto plates to capture samples. Then, a rabbit serum was incubated before  
851 detection with Goat polyclonal Secondary Antibody to Rabbit IgG - H&L (HRP) (Abcam Cat#  
852 ab97080, RRID:AB\_10679808). The plate development, data acquisition, and analysis were the  
853 same as described above.

854

### 855 *Size-exclusion ultra-performance liquid chromatography (SE-UPLC)*

856 Size exclusion chromatography of modified mRNA-expressed GNL-purified CH848 10.17DT  
857 SOSIP trimers was performed using a Waters Acquity H-Class Bio UPLC System with a Waters  
858 Acquity UPLC BEH SEC 450Å, 2.5 µm, 4.6 x 150 mm column (Waters Corporation). An isocratic  
859 elution with a mobile phase of 20 mM sodium phosphate 300 mM NaCl pH 7.4, and a flow rate of  
860 0.2 ml/min, was used for the analysis with a quaternary pump. Samples and protein standards  
861 were maintained at 5-8°C in the auto-sampler rack prior to injection at a volume of 10 µl. Samples  
862 and protein standards with a concentration greater than 1.0 mg/ml were diluted to a down to 1.0  
863 mg/mL using Type 1 water. The column temperature was set to 30 °C with detection at a  
864 wavelength of 214 nm using a photodiode array detector.

865

#### 866 *Negative-stain Electron Microscopy (NSEM)*

867 Negative-stain electron microscopy (NEM) analysis of modified mRNA-expressed CH848  
868 10.17DT SOSIP trimers and trimer-ferritin NPs were performed as previously described  
869 ([Saunders et al., 2017](#); [Williams et al., 2021](#)).

870

#### 871 *Surface Plasmon Resonance (SPR)*

872 SPR analyses of modified mRNA-expressed SOSIP proteins incubated with and without sCD4  
873 against distal V3 loop antibody 19b and CCR5 binding site antibody 17b were obtained using the  
874 Biacore S200 instrument (Cytiva). Antibodies 19b and 17b were immobilized onto a CM3 sensor  
875 chip to a level of 2000-4000RU. A negative control Influenza IgG1 antibody (CH65) was also  
876 immobilized onto the sensor chip for reference subtraction. Modified mRNA-expressed GNL-  
877 purified CH848 10.17DT SOSIP trimers or trimer-ferritin NPs were diluted down in HBS-N 1x  
878 running buffer to 0.5-2.0 µg and incubated with a 2-8x higher dose of soluble CD4 (4.4 µg)  
879 (Progenics Therapeutics). Proteins incubated with and without sCD4 were injected over the  
880 sensor chip surface using the High performance injection type for 180s at 30 µl/min. The protein  
881 was then allowed to dissociate for 600s followed by sensor surface regeneration of two 20 s

882 injections of glycine pH 2.0 at a flow rate of 50  $\mu$ l/min. Results were analyzed using the  
883 BIAevaluation Software (Cytiva). Protein binding to the CH65 immobilized sensor surface as well  
884 as buffer binding were used for double reference subtraction to account for non-specific protein  
885 binding and signal drift.

886

### 887 **Mouse serological analysis by ELISA**

#### 888 *Serum IgG antigen binding assay*

889 Serum IgG binding to HIV-1 antigens was measured by ELISA as previously described ([Saunders](#)  
890 [et al., 2019](#)).

#### 891 *DH1029 blocking assay*

892 DH1029 blocking by vaccinated mouse sera was performed in ELISA. Briefly, 384-well assay  
893 plate were coated with 2  $\mu$ g/ml PGT145. Then, 0.125  $\mu$ g/ml of CH848 10.17DT SOSIP trimer were  
894 captured for 1 h at room temperature. Next, mouse sera at 1:50 dilution or DH1029 mAb in serial  
895 dilution were incubated for 1 h. Next, biotinylated DH1029 were added to the plate for 1 h and  
896 binding were detected by High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific, Cat  
897 #21130). Plate development and data acquisition were the same as described above.

898

### 899 **HIV-1 pseudovirus neutralization assay**

900 Neutralization assays were performed in TZM-bl reporter cells as described ([Mascola et al.,](#)  
901 [2005](#)).

902

### 903 **Next-generation sequencing (NGS)**

904 We performed next-generation sequencing (NGS) on mouse antibody heavy and light chain  
905 variable genes using an Illumina sequencing platform. First, RNA was purified from splenocytes  
906 using a RNeasy Mini Kit (Qiagen, Cat# 74104). Purified RNA was quantified via Nanodrop  
907 (Thermo Fisher Scientific) and used to generate Illumina-ready heavy and light chain sequencing

908 libraries using the SMARTer Mouse BCR IgG H/K/L Profiling Kit (Takara, Cat# 634422). Briefly,  
909 1 µg of total purified RNA from splenocytes was used for reverse transcription with Poly dT  
910 provided in the SMARTer Mouse BCR kit for cDNA synthesis. Heavy and light chain genes were  
911 then separately amplified using a 5' RACE approach with reverse primers that anneal in the  
912 mouse IgG constant region for heavy chain genes and IgK for the light chain genes (SMARTer  
913 Mouse BCR IgG H/K/L Profiling Kit). The DH270 UCA KI mouse model has the light chain gene  
914 knocked into the kappa locus, therefore kappa primers provided in the SMARTer Mouse BCR kit  
915 were used for light chain gene library preparation. 5 µl of cDNA was used for heavy and light  
916 chain gene amplification via two rounds of PCR; PCR1 used 18 cycles and PCR2 used 12 cycles.  
917 During PCR2, Illumina adapters and indexes were added. Illumina-ready sequencing libraries  
918 were then purified and size-selected by AMPure XP (Beckman Coulter, Cat# A63881) using kit  
919 recommendations. The heavy and light chain libraries per mouse were indexed separately, thus  
920 allowing us to deconvolute the mouse-specific sequences during analysis. Libraries were  
921 quantified using QuBit Fluorometer (Thermo Fisher). Mice were pooled by groups for sequencing  
922 on the Illumina MiSeq Reagent Kit v3 (600 cycle) (Illumina, Cat# MS-102-3003) using read lengths  
923 of 301/301 with 20% PhiX.

924

### 925 **Antibody sequence analysis**

926 NGS data analysis and the analysis of improbable mutation frequencies was performed as  
927 described ([Wiehe et al., 2018](#)).

928

### 929 **Flow cytometric phenotyping of GC responses**

930 For immunophenotyping of murine B cells and Tfh cells, spleens from immunized mice one  
931 week after the third immunization were processed into single-cell suspensions and treated with  
932 ACK lysis buffer to remove red blood cells. Splenocytes ( $2 \times 10^6$ ) were suspended in 100 µL PBS/2%  
933 FBS. To detect antigen-specific B cells, fluorochrome-mAb conjugates and fluorochrome-

934 conjugated CH848 10.17DT Envs were prepared as a master mix at 2x concentration, then 100  
935  $\mu$ L of 2x master mix was added to an equal volume of cells (**Figure S4**). Staining for T cell subsets  
936 was conducted in the same manner, with the additional step for detection of biotinylated mAb with  
937 Streptavidin–APC. Cells were incubated at 4°C for 20 minutes, then washed with PBS. Cells were  
938 resuspended in 100  $\mu$ L PBS containing Near-IR Live/Dead (Thermo Fisher Scientific) at 1:1000,  
939 and incubated at room temperature for 20 min. Cells were washed in PBS/2% FBS, then re-  
940 suspended in PBS/2% formaldehyde. Cells were analyzed on a BD LSRII (BD Biosciences). Data  
941 were analyzed using FlowJo v10 (FlowJo).

942

### 943 **Isolation of CH848 10.17DT-specific neutralizing monoclonal antibodies (mAbs)**

#### 944 *Antibody cloning, screening, and mAbs expression*

945 Immunoglobulin (Ig) gene were cloned from sorted single B cells as previously described ([Liao et](#)  
946 [al., 2009](#)). Briefly, complementary DNA (cDNA) of Ig genes were amplified by reverse-  
947 transcription with SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Cat#  
948 18080051) using random hexamer oligonucleotides as primers. Ig gene cDNA was then used as  
949 template in nested PCR for heavy and light chain gene amplification using AmpliTaq Gold 360  
950 Master Mix (Thermo Fisher Scientific, Cat #4398881). Mouse Ig-specific primers and DH270  
951 variable region-specific primers were used to amplify mouse endogenous Ig genes and DH270  
952 KI Ig genes. Agarose gel electrophoresis was used to identify positive PCR amplification and Ig  
953 genes were recovered by Sanger sequencing. Following sequencing, contigs of PCR amplicon  
954 sequences were assembled, and Ig genes were inferred with human Ig gene library and mouse  
955 Ig gene library in Cloanlyst. PCR reactions with successful Ig sequence recovery were purified  
956 using AMPure XP kit (Beckman Coulter, Cat# A63881). Purified PCR product was used for  
957 overlapping PCR to generate a linear antibody expression cassette. The expression cassette was  
958 transiently transfected with into 293i cells with ExpiFectamine 293 Transfection Kit (Thermo  
959 Fisher Scientific, Cat# A14525). The supernatant was harvested 72 h after transfection and

960 screened in ELISA binding assays with a panel of protein of interests. The genes of selected  
961 heavy chains were synthesized with human IgG1 backbone (GenScript). Kappa and lambda  
962 chains were synthesized similarly. To express mAbs plasmids were prepared for transient  
963 transfection using the Plasmid Plus Mega Kit (Qiagen, Cat #12981). Heavy and light chain  
964 plasmids were co-transfected into 293i cells using ExpiFectamine 293 Transfection Kit for  
965 antibody production.

966

### 967 **QUANTIFICATION AND STATISTICAL ANALYSIS**

968 Exact Wilcoxon Mann-Whitney U tests were performed without any adjustment for multiple  
969 comparisons. Significant results were indicated in figures and figure legends as: \*  $P < 0.05$ ; \*\*  $P <$   
970  $0.01$ .

971

972 **Table S3. CH848 10.17DT trimer-ferritin NP mRNA-LNP vaccine-induced monoclonal**  
973 **antibodies ELISA binding magnitudes. Related to Figure 7.**

974

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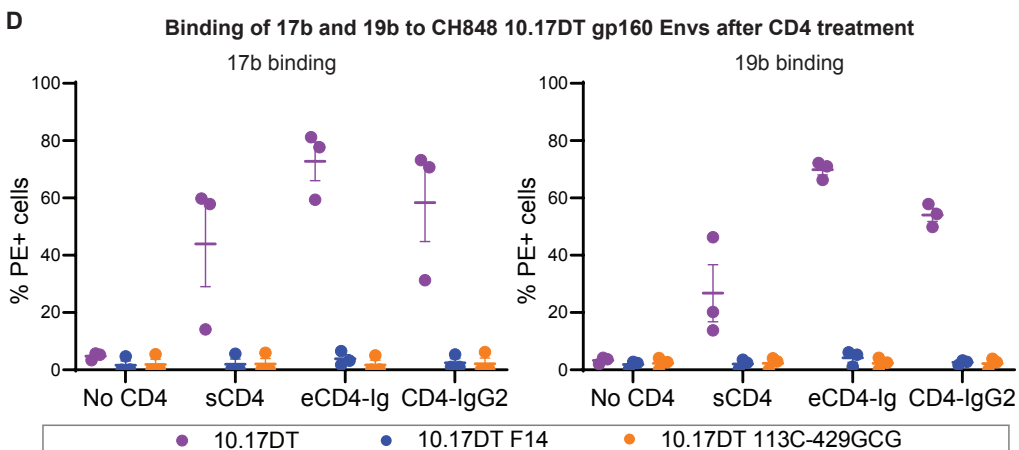
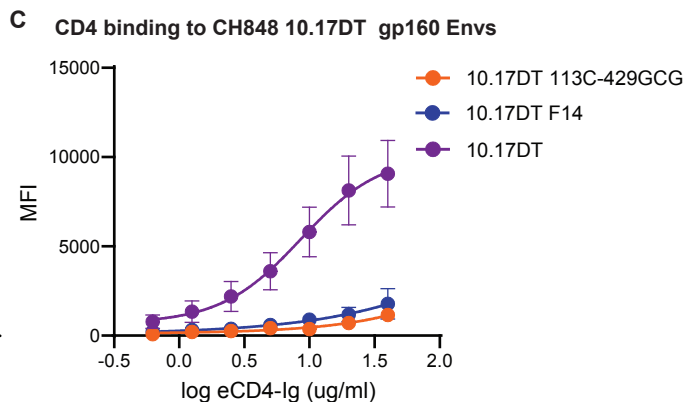
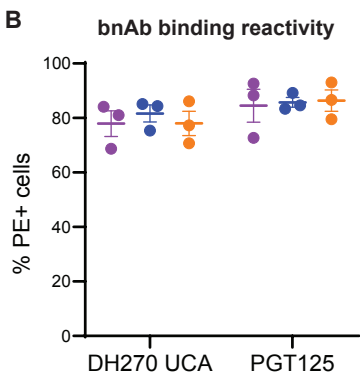
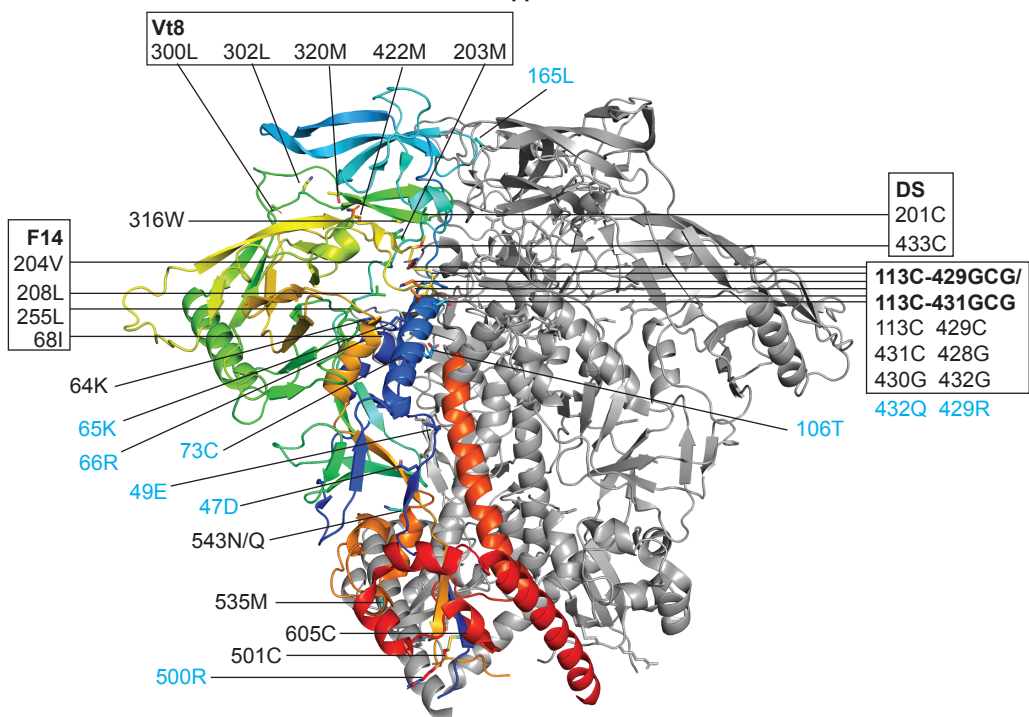
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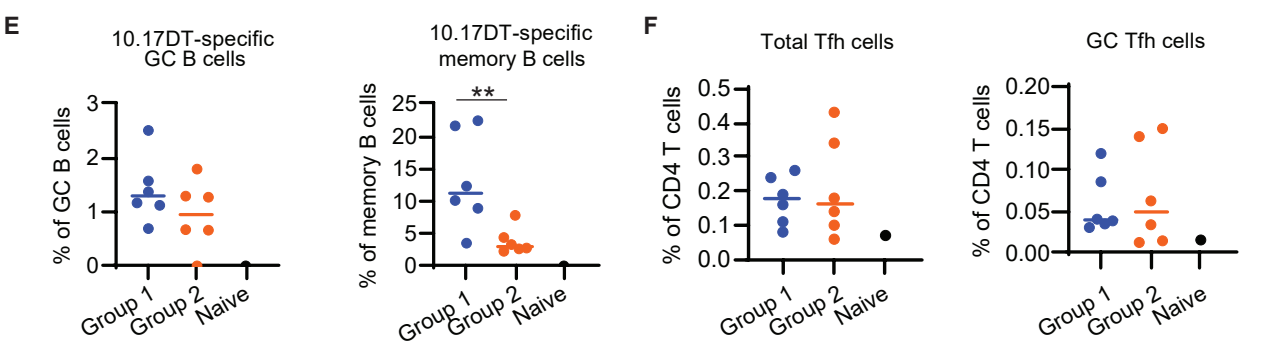
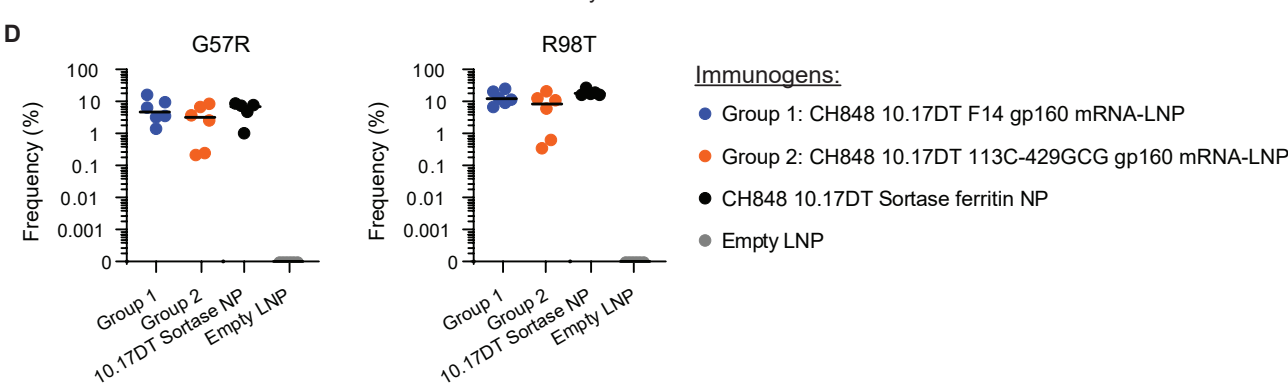
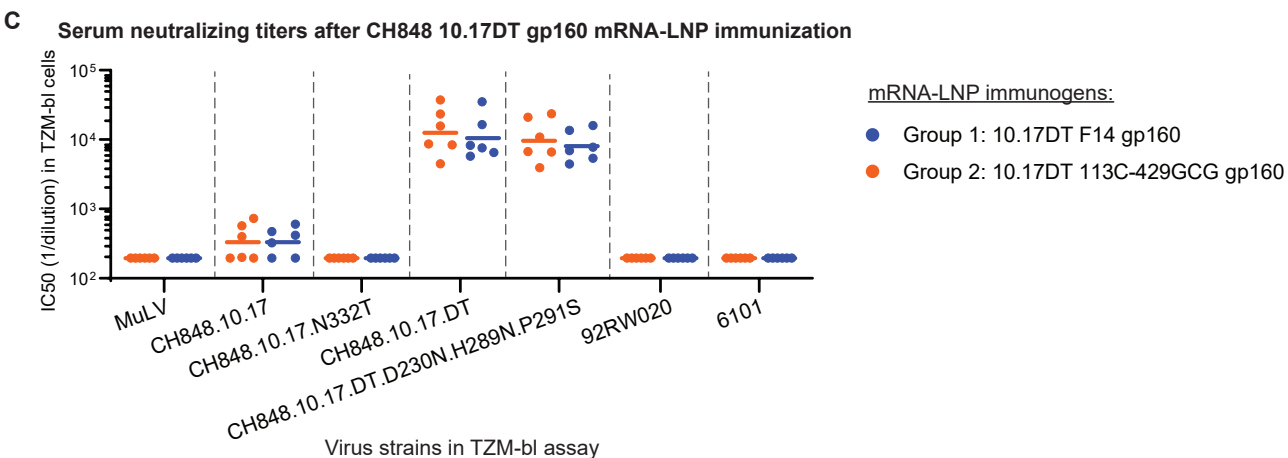
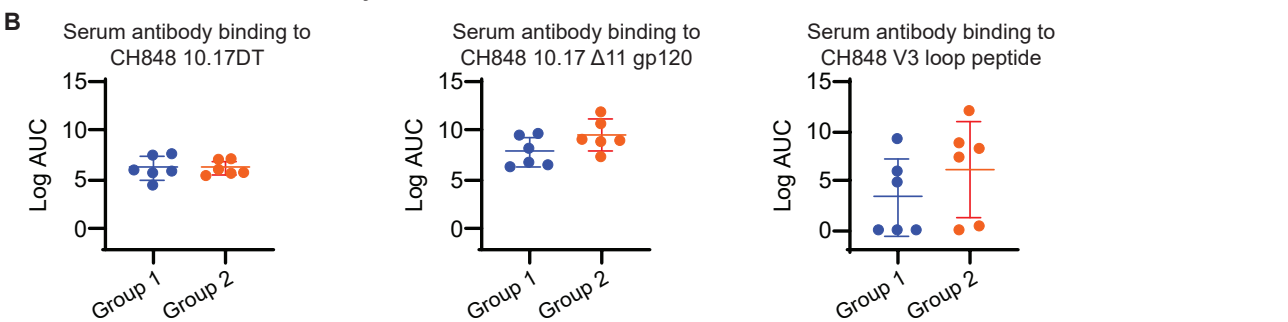
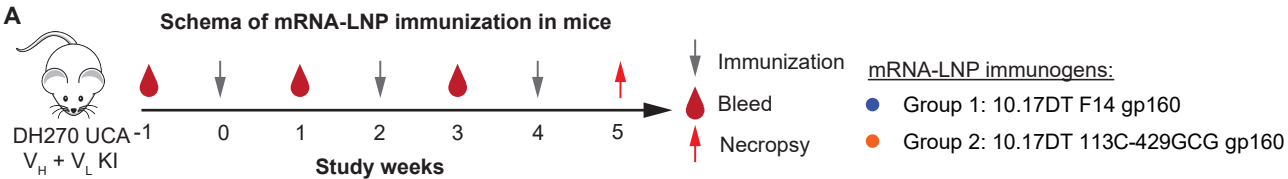
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1181

### Stabilization mutations tested mapped on CH848 10.17DT SOSIP trimer





A

## Binding reactivities of bnAbs and nnAbs to modified mRNA-expressed CH848 10.17DT SOSIP trimers

nnAbs

bnAb/bnAb precursors

Trimers	nnAbs									bnAb/bnAb precursors									
	coreceptor			V3	V2		CD4bs		CD4i	DH270 lineage			V3-glycan			V2-glycan			gp41-gp120 interface
	17b	19b	F39F	CH58	697D	F105	b12	A32	DH270 UCA	DH270 IA4	DH270.1	2G12	PGT125	PGT128	PGT145	CH01	PG9	VRC26.25	PGT151
v4.1	1	2	1	0	3	3	3	1	4	4	4	3	3	7	1	1	1	0	6
DS	0	2	3	0	3	4	1	2	4	4	4	3	3	6	1	2	1	0	6
F14	0	1	1	0	3	1	1	1	3	3	3	2	2	5	1	1	1	0	5
Vt8	0	1	0	0	1	2	1	1	2	2	3	2	5	6	1	1	1	0	4
F14/Vt8	0	1	0	0	2	1	1	1	1	2	3	2	5	6	1	1	1	0	3
v5.2.8	1	3	1	0	2	6	1	0	3	3	3	2	2	5	0	1	1	0	5
v5.2.8+UFO	0	1	0	0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0

ELISA score	0	1	2	3	4	5	6	7
logAUC	=0	0-1	1-2	2-3	3-4	4-5	5-6	6-7

B

## SPR measurement of 17b and 19b binding to modified mRNA-expressed CH848 10.17DT SOSIP trimer after sCD4 treatment

17b

19b

17b

19b

10.17DT SOSIPv4.1

10.17DT F14/Vt8 SOSIP

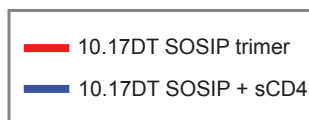
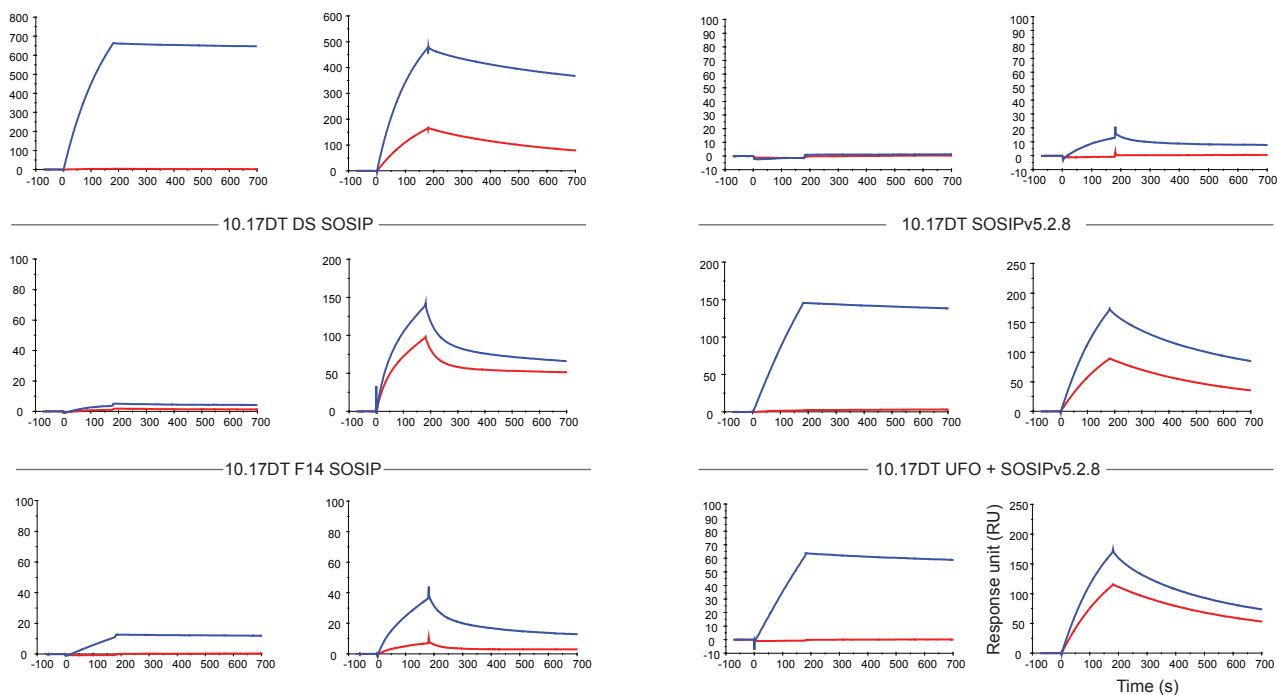
10.17DT DS SOSIP

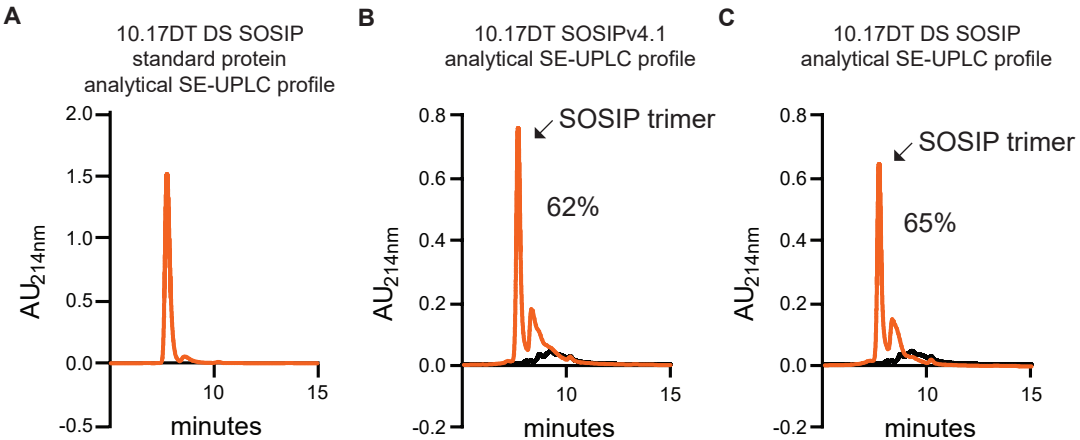
10.17DT SOSIPv5.2.8

10.17DT F14 SOSIP

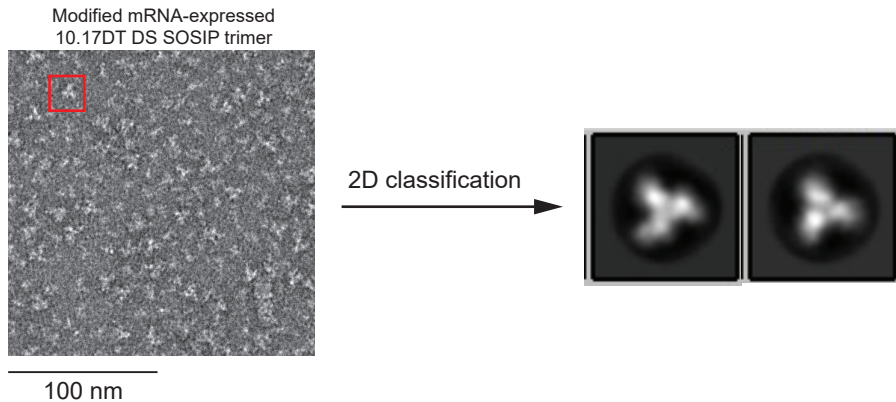
10.17DT UFO + SOSIPv5.2.8

10.17DT Vt8 SOSIP



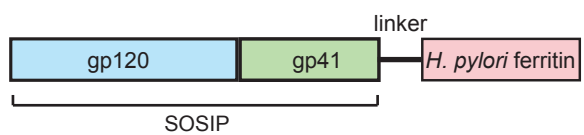


**D** NSEM analysis of modified mRNA-expressed CH848 10.17DT DS SOSIP trimer





# A Design of CH848 10.17DT trimer-ferritin nanoparticles



## B

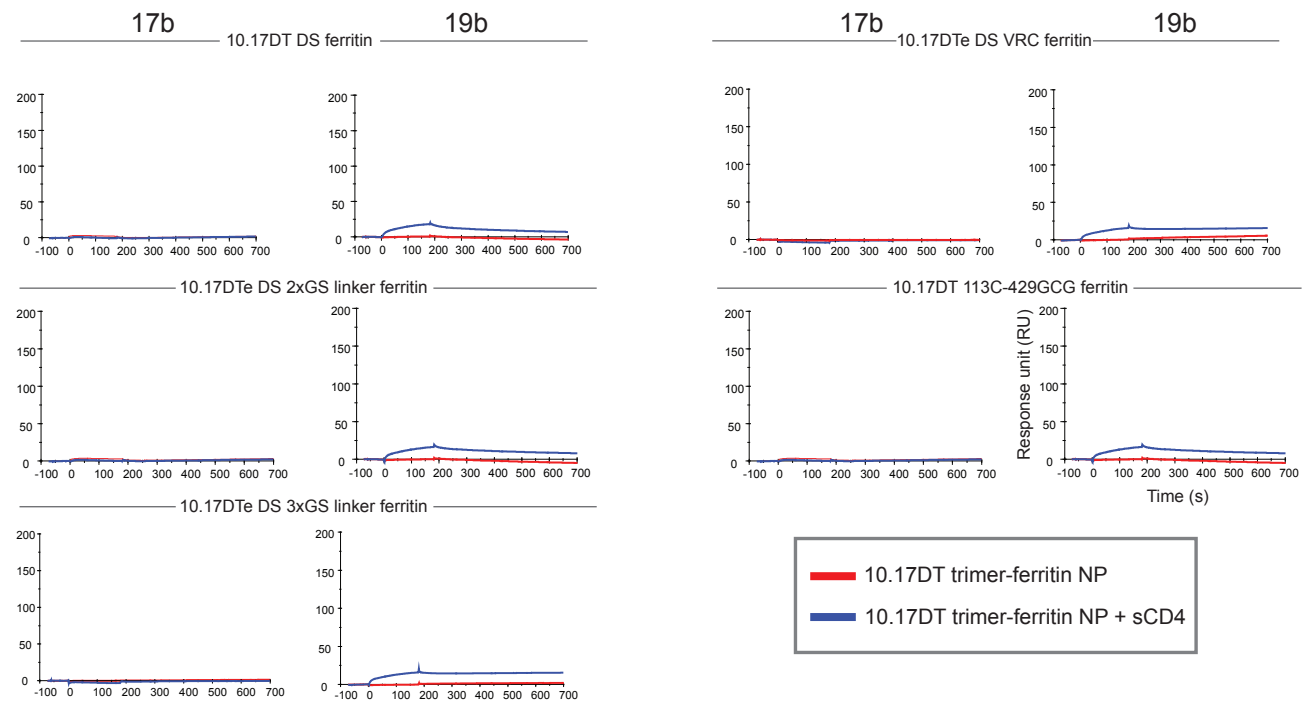
### Binding reactivities of bnAbs and nnAbs to modified mRNA-expressed CH848 10.17DT trimer-ferritin NPs

Trimer-ferritin NPs	nnAbs								bnAbs/bnAb precursors										
	coreceptor		V3	V2		CD4bs		CD4i	DH270 lineage			V3-glycan			V2-glycan		gp41-gp120 interface		
	17b	19b	F39F	CH58	697D	F105	b12	A32	DH270 UCA	DH270 IA4	DH270.1	2G12	PGT125	PGT128	PGT145	CH01	PG9	VRC26.25	PGT151
10.17DT DS	0	2	1	0	1	3	0	1	2	3	3	2	3	4	0	1	1	0	3
10.17DTe DS 2xGS linker	0	2	1	1	1	2	0	2	2	3	3	3	4	5	4	2	5	5	3
10.17DTe DS 3xGS linker	0	1	1	1	1	1	0	1	2	2	3	3	3	5	3	1	4	4	2
10.17DTe DS VRC	0	2	2	1	1	3	0	2	1	1	2	2	2	4	1	1	4	3	1
10.17DT 113C-429GCG	0	5	5	0	2	1	2	1	2	2	3	2	3	5	0	0	0	0	3

ELISA score	0	1	2	3	4	5
logAUC	=0	0-1	1-2	2-3	3-4	4-5

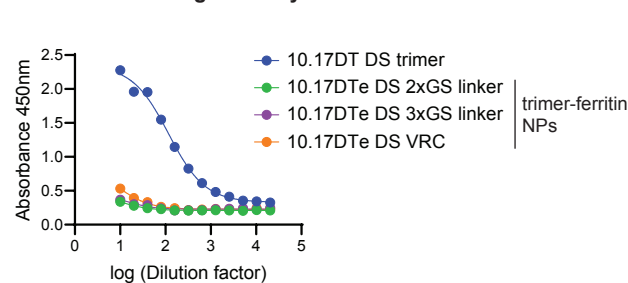
## C

### SPR measurement of 17b and 19b binding to modified mRNA-expressed CH848 10.17DT trimer-ferritin NPs after sCD4 treatment



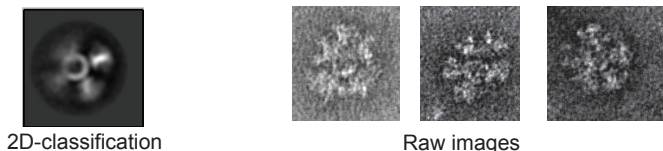
## D

### Base binding antibody DH1029

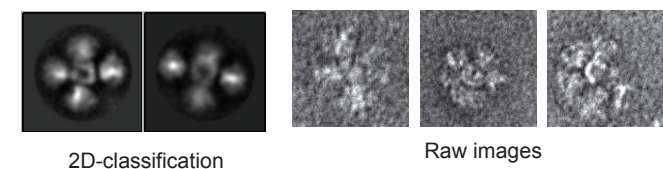


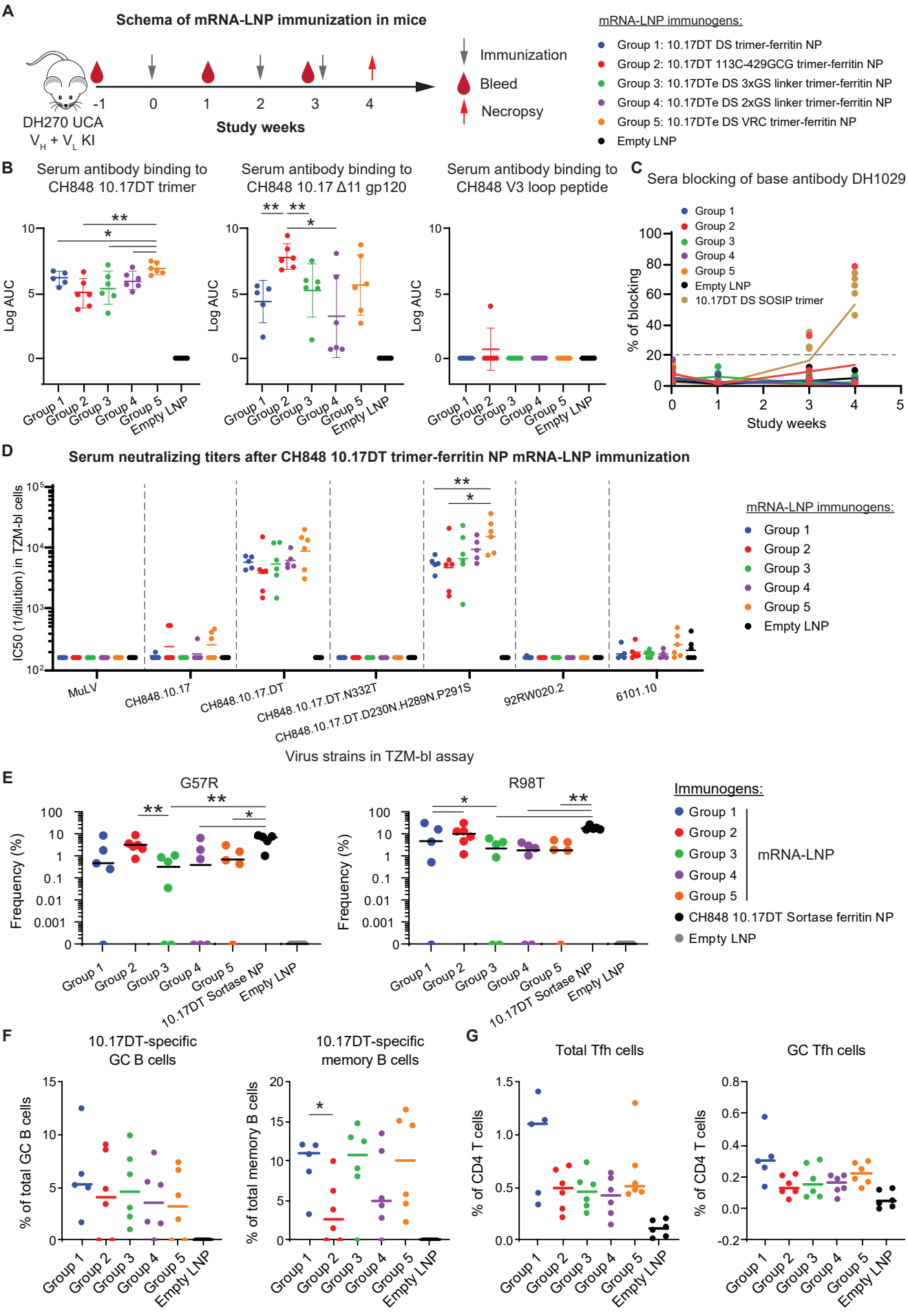
## E

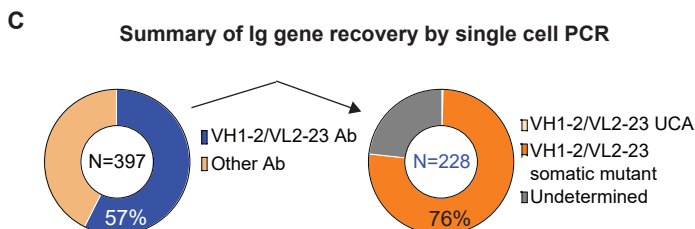
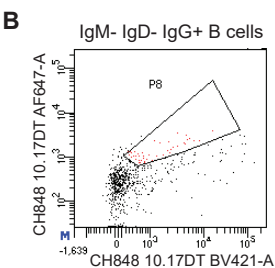
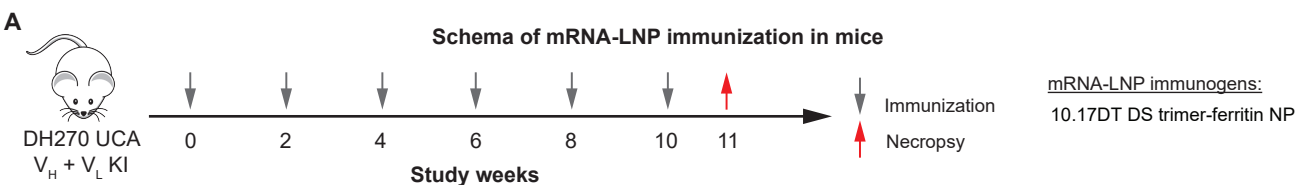
### 10.17DTe DS VRC trimer-ferritin NP



### 10.17DTe DS 3xGS linker trimer-ferritin NP







**D** **VH1-2/VL2-23 Abs with improbable mutations**

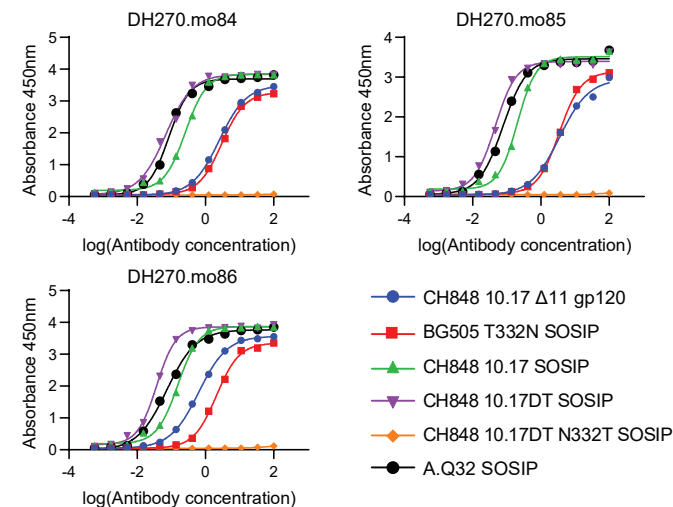
Heavy chain

G57R	R98T
5 (2%)	20 (9%)

Light chain

L48Y
2 (1%)

**E** **Antibody binding reactivity**



**F** **Antibody neutralization activities**

Viruses	CH848.d949.10.17.N133D.N138T	<0.023	<0.023	<0.023	IC50 (μg/ml)
CH848.d949.10.17	0.104	0.09	0.06		
92RW020	0.367	1.886	0.45		
6101.1	0.84	1.93	7.3		>50
6535	0.905	2.41	15.18		5-50
92BR025	0.905	7.28	7.79		0.5-5
CNE14	3.345	5.143	2.77		0.05-0.49
398-F1-F6_20	4.33	30.14	1.25		<0.05
SF162.LS	3.164	11.34	34.54		
XB08.16	11.02	>50	30.7		
P1981_C5_3	5.46	20.26	4.33		
CNE12	32.4	>50	27.66		
Q23	34.68	>50	>50		
45_01dG5	34.63	>50	12.376		
TRO.11	15.124	>50	>50		
T280-5	4.821	7.72	1.87		
ZM55F.PB28a	25.482	46.75	>50		
MJLV	>50	>50	>50		

**G** **Improbable mutations in selected mAbs**

